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Under the Editorship of LUDVIK HEKTOEN and EDWIN O. JORDAN

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AN EVALUATION OF ANTIMALARIALS WITH *PLASMODIUM LOPHURAE* IN THE CHICK

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The purpose of the experiments here reported was to obtain quantitative data on the suppressive potency of commonly used antimalarials when administered under controlled conditions to chickens infected with *Plasmodium lophurae*. The data thus obtained may serve as a basis for comparison of newer antimalarials when these drugs are used either singly or in combinations. An assay procedure for antimalarial activity is given in this communication; the results of the joint effect of antimalarials will be reported in a subsequent paper.

MATERIALS AND METHODS

Parasite.—The strain of *P. lophurae* used in the experiments was isolated by Coggeshall from *Lophurae igniti igniti* in 1938. This strain was obtained from the Johns Hopkins Medical School through the courtesy of Doctor E. K. Marshall, Jr. The parasite was transferred from young Pekin ducklings into week-old Leghorn chicks and was kept at an infective level in this laboratory by blood transfers every 4 to 6 days for 3 years. The strain had less than 1% gametocytes when used in these tests. It is, therefore, suitable for testing drugs against the asexual stages. The intensity of the infection can be regulated by varying the frequency of transfers.[1]

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† Research Laboratories, Parke Davis and Company.

Avian host—Young white Leghorn cockerels of pullorum-free strain were used in these tests. They were received at the age of three days and maintained on an alternating three-hour light and dark schedule. The chicks were fed "chick starter," and those weighing between 50 and 60 g at the age of 8 to 9 days were selected for use. Out of 150 chicks, there were approximately 100 within the weight range selected.

Housing of chicks.—The young chicks were kept in two-shelf cages with floor dimensions of 24×26 inches, each of which was divided in half. To ensure proper lighting, which is very important in the drug diet method, a 150 watt bulb was placed near each cage and uniform light intensity adjusted in the cage with the aid of a photocell. Four 150 watt bulbs were used overhead. The temperature of the room was maintained at 90 F. A continuously operating fan insured adequate ventilation of the room.

Inoculum.—The inoculum was obtained by heart puncture from chicks during the acute rise of the infection at a time when 10 to 25% of the blood cells were parasitized. The blood from each donor bird was drawn into 0.4 cc of a 1% solution of heparin and diluted with oxygenated Ringer-Locke solution to make an average of 50×10^6 parasitized cells per 0.2 cc. This number was calculated by converting percent of cells parasitized to the number of parasitized cells. Haemocytometer counts on young chicks showed that there were approximately 3,000,000 red blood cells per cmm. This normal haemocytometer count was multiplied by the percent of cells parasitized to give the number of cells parasitized per cmm. The red cell count was not appreciably reduced at the time when infected chicks were selected for transfer.

Procedure of the tests.—From 90 to 120 chicks, weighing between 50 and 60 g, were used in each of the experiments. The following procedure was used in selecting chicks of uniform weight for the test groups: The chicks were divided into 5 groups. Group 1 consisted of the heaviest chicks, group 2 the lightest ones, and the remainder were distributed among the other three groups. The heaviest ones were infected first and the lightest ones last, and placed back in their respective

1. Terzian, L. 1941, Am. J. Hyg. **33** (Sec. C): 1–22.

groups. The inoculum, 0.2 cc of blood containing 50×10^6 parasitized cells per chick, was injected into the jugular vein. The injections were usually completed within 45 to 60 minutes.

After the inoculation was finished the chicks were distributed into 10 or more test cages. The heaviest birds were distributed first and the lightest ones last. The groups composed of 8 to 10 chicks per test cage were then reweighed and banded. The average weight of any single group did not deviate from the average weight of all of the groups by more than 1 or 2 g.

The chicks were fed regular "chick starter" for the first 12 hours and were then fed the particular diet to be tested for 5 days. Blood smears were made on the 3rd, 4th and 5th days and the chicks were then discarded.

Drug administration.—Quinine hydrochloride, quinacrine dihydrochloride, pamaquine hydroiodate and pentaquine diphosphate were used in these tests. All data are expressed in terms of the drug weighed as free base. The drug was weighed, ground with 20 g of dextrose and mixed with 1300 g of chick starter. This mixture was placed in one gallon glass jars which were rotated, usually for 6 hours. As previously mentioned, chicks were fed the mixed drug-diet from 12 hours after inoculation. The diet bins were protected by small rotating guards which enabled the chicks to eat freely but prevented excessive spilling of food.

From calculations based on diet consumption records, which were kept on each group of birds for the duration of the test, each chick ordinarily consumed about 10 g of "starter" daily. A few chicks, however, failed to eat, especially as the parasitemia approached its peak. Since data with respect to the suppressive action of a given drug were taken on the 4th day, anorexia which developed after the 4th day did not affect the results.

Drug dosage.—Tests were performed with different concentrations of quinine, quinacrine, pamaquine and pentaquine for 5 days beginning 12 hours after infection. On the basis of previous screening tests conducted in this laboratory during World War II, concentrations of a drug were selected which suppressed the parasitemia by 10 to 90% of that of the control birds.

Staining and counting the parasites.—Blood smears were air-dried and stained with MacNeil's tetrachrome stain. The slides were examined according to the method of W. H. and L. G. Taliaferro.[2]

Mode of expressing data.—Data are expressed as percentage of red blood cells parasitized (% C. P.), since Wolfson and Cakrtova[3] have shown that multiple parasitized cells increase uniformly as the parasitemia increases. The mean parasite count for each group of birds was determined on the 4th day, and that of the controls was considered as zero suppression of the parasitemia.

The percentage of cells parasitized was converted to percentage suppression of the parasitemia according to the following formula:

$$\frac{\%\text{ C. P. controls} - \%\text{ C. P. treated}}{\%\text{ C. P. controls}} \times 100 = \%\text{ suppression of parasitemia}$$

The percentage suppression of parasitemia in terms of probits was then plotted against the logarithm of the dose of the drug.[4] From the linear relationship, the concentration of a drug in the diet necessary to suppress the parasitemia to 50% of that of the control group (SD_{50}) was interpolated at probit 5. The 50% suppressive dose compared with that of quinine was used, rather than the minimal effective dose,[5,6] in evaluating the activity of antimalarials.

RESULTS AND DISCUSSION

Preliminary experiment to determine the size of the inoculum.—This experiment was designed to ascertain the number of parasites needed as an inoculum to obtain an acute and rapid rise of parasitemia during which innate immunity but little acquired immunity was present. Six groups of 10 birds each were inoculated intravenously with 12×10^6, 25×10^6, 50×10^6, 75×10^6, 100×10^6 and 125×10^6 parasitized cells. The data are represented by curves in figure 1. An infection of 50×10^6 was selected as the optimum for the succeeding tests because in 8-day-old-chicks weighing between 50 to 60 g it gave rise to a parasitemia which rose progressively through at a slightly decreasing rate until 20 to 30% of the

2. Taliaferro. W. H. and Taliaferro. L. G., 1940, 1940. J. Infect. Dis. **66:** 153–165.

3. Wolfson, F. and Cakrtova, M. 1946, Am. J. Hyg. **44:** 301–311.

4. Bliss, C. I. 1935, Ann. Applied Biol. **22:** 134–167.

5. Marshall, P. B. 1945, J. Pharmacol. & Exper. Therap. **85:** 299–309.

6. Gingrich, W. D. and Schoch, E. W. 1947, J. Nat. Malaria Soc. **6:** 68.

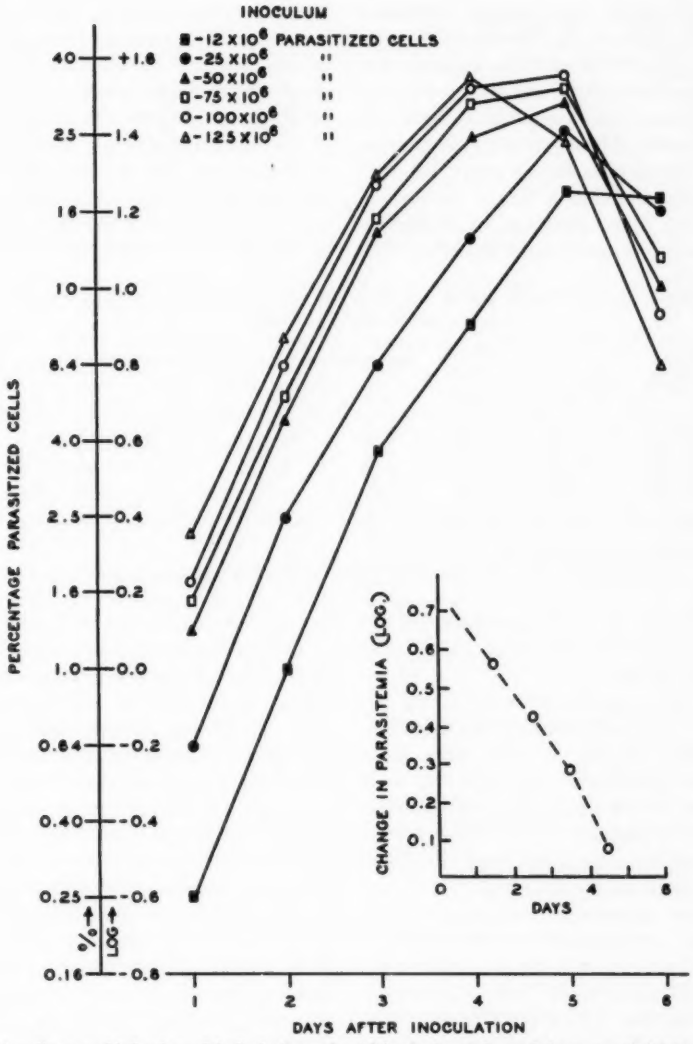

FIG. 1.—Course of infection with *P. lophurae* in chicks during 6 days of the patent initial infection. The inset graph shows average values of all inoculations (in logarithms) for the daily change in parasitemia and for the first 24 hours by extrapolation. Note that these values indicate that the change in rate is consistent during the first 4 days.

cells were parasitized on the 4th day. The steepness of the slope of the parasitemia curve was greatly decreased from the 4th to the 5th day of the test at the 50×10^6 level. This suggests that the immunity acquired during this period caused an appreciable break in parasitemia. However, a much sharper break in the parasitemia curve occurred in birds receiving a larger number of parasites. The question of immunity will be discussed subsequently. The ulum. After the 4th day, however, there appears a variation in rate directly proportional to the size of the inoculum. The average change in rate of parasitemia of all inoculations is graphically represented in figure 1 (inset graph). From the linear portion of the curve, the average rate of increase in parasitemia for the first 24 hours is obtained by extrapolation. This value is used in the following calculations of the actual number of parasitized cells in the

TABLE 1.—*Over-all daily difference in the parasitemia (in logarithms) of* P. lophurae *through the 6th day of infection.*

Dose of parasites inoculated	Difference in parasitemia (in logarithms)							
	a* 1–2	b 2–3	c 3–4	d 4–5	e 5–6	a–c	a–d	a–e
12×10^6	0.60	0.57	0.34	0.34	−0.01	0.26	0.26	0.61
25×10^6	0.60	0.41	0.33	0.27	−0.20	0.27	0.33	0.80
50×10^6	0.55	0.50	0.25	0.09	−0.39	0.30	0.46	0.94
75×10^6	0.54	0.47	0.30	0.03	−0.34	0.24	0.51	0.88
100×10^6	0.57	0.48	0.25	0.03	−0.62	0.32	0.54	1.19
125×10^6	0.53	0.42	0.26	−0.27	−0.59	0.27	0.80	1.12
† Average ± S.E.	0.56 ± 0.01	0.44 ± 0.03	0.29 ± 0.02	0.08	−0.36	0.28	0.48	0.99

t: $a-b=6.79$, $p<0.01$; $b-c=13$, $p<0.01$.
* From the end of the first day to the end of the second day of infection, etc.
† Average: b_{t_1}, b_{t_2}, b_{t_3}, b_{t_4} (antilog of 0.70, 0.56, 0.44, 0.29) = 5.01, 3.63, 2.75 and 1.95, respectively.

12×10^6 and 25×10^6 infections did not give a sufficient degree of parasitemia on the 4th day.

Another experiment was carried out to test the uniformity of the infection. For this purpose 90 birds were given 50×10^6 parasitized red cells and were divided into 9 test groups of 10 birds each. The parasitemias of these groups deviated by only 10% from the mean parasitemia of the 90 birds.

Innate immunity and acquired immunity.—An inspection of the curves in figure 1 reveals that the logarithmic increase in parasitemia diminishes gradually from the 1st to the 4th day. The average values in logarithms of the daily change in parasitemia, given in table 1, indicate that the difference in rate is statistically significant and consistent during the first 4 days. Furthermore, it is the same for infections initiated with different doses of the inoc- circulation at the start of the infection.

The two types of defense mechanism of the host in combating an infection, innate immunity and acquired immunity, are clearly demonstrated in this experiment. The former is present at the beginning of the infection during which a certain portion of the parasites disappear. The simple exponential formula $y=ab^t$, where y = parasitemia at t days after inoculation, a = initial parasitemia, b = the daily fractional increase in parasitemia and t = time since inoculation in days, is only an approximation for the increase in parasitemia before acquired immunity begins to play a major part in the defense mechanism. In our experiment, the empirical formula was found to be $y=a(b_{t_1}b_{t_2}b_{t_3}b_{t_4})$. In this formula, b_{t_1}, etc. represent the average daily rate of increase of parasitized cells for the 1st, 2nd, 3rd and 4th day of infection. For example, the average

TABLE 2.—*Influence of the size of inoculum on the initial parasitemia, percent parasitized cells (4th day) and eliminated cells (total at the end of the 4th day).*

Dose of parasites inoculated	y (1st day) Observed	a*	Percent parasitized cells (4th day)		Percent eliminated parasitized cells (total) at end of 4th day)‡
			Calculated†	Observed	
12×10^6	0.25	0.05	4.9	8.1	1.46
25×10^6	0.61	0.12	11.7	13.8	3.52
50×10^6	1.25	0.25	24.3	25.3	7.32
75×10^6	1.50	0.30	29.3	31.0	8.79
100×10^6	1.70	0.34	33.2	34.0	9.96
125×10^6	2.25	0.45	44.0	36.3	13.19

* a calculated from $y=a\,b_{t_0}\,b_{t_1}$ (first 24 hours) $=5.01$ (antilog of 0.70).
† y calculated from $y=a(b_{t_1}\,b_{t_2}\,b_{t_3}\,b_{t_4})=a(5.01)\ (3.63)\ (2.75)\ (1.95)=97.72\,a$.
‡ Eliminated cells $=a(b_t-b_{t_4})+b_{t_3}(b_1-b_{t_3})+b_{t_2}\,b_{t_3}(b_2-b_{t_3})+b_{t_1}\,b_{t_2}\,b_{t_3}(b_3-b_{t_4})=29.3a$.
b_0, b_1, b_2, b_3 = rate of increase of percent parasitized cells at the beginning of each day.

parasitemias of chickens receiving 50×10^{-6} parasitized cells as inoculum was 1.35%, 4.6%, 14.5% and 25% parasitized cells for the first 4 days of the patent infection, which gave corresponding logarithmic values of 0.1, 0.65, 1.15 and 1.4 (fig. 1). When the differences in parasitemia (in logarithms) were then calculated for each group of chickens and averaged daily, the resulting averages were found to be 0.56, 0.44, 0.29 and 0.08 from the end of the 1st through the 5th day (table 1). These average values were then plotted and the curve extrapolated to zero time, giving a logarithmic value of 0.7, which was considered as the value for the average difference in parasitemia during the first 24 hours (inset graph, fig. 1). This value, 0.7, was used in the calculations of the actual number of parasitized cells in the circulation at the start of the infection and in determining the number of cells eliminated at the end of a given time. The antilogs of 0.70, 0.56, 0.44 and 0.29 were used in the formula representing b_{t_1}, b_{t_2}, b_{t_3} and b_{t_4}, respectively. The b_t values were used in calculating the initial parasitemia, the parasitemia at t days after inoculation and the number of cells eliminated at the end of a given time (table 2). The b values, however, have no precise significance in the reproductive division of parasites. If it is assumed that the rate of reproduction is not impaired by innate immunity,[7,8] a constant decrease in b values during the first 4 days signifies a gradual increase in the destruction of parasites by other mechanisms. These values were based on counts of parasitized cells and would no doubt have

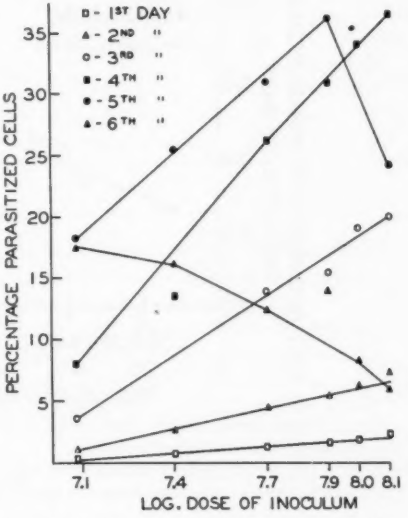

FIG. 2.—Relationship of increased size of inoculum to the daily change in percentage parasitized cells.

Note that the curves are practically linear with an increasing slope through the first 4 days, and that the slope decreases on the 5th day and reverses its direction on the 6th day.

7. Taliaferro, L. G. 1925, Am. J. Hyg. **5**: 742–789.
8. Taliaferro, W. H. 1948, Bact. Rev. **12**: 1–17.

been slightly different had actual parasite counts been used.

An interesting relationship is also obtained by plotting the daily count of the percentage of parasitized cells against the log dose of the inoculum (fig. 2). This is practically linear for the 1st through the 4th day. For the 5th day, the slope decreases and for the 6th day reverses its direction. This indicates that acquired immunity was manifested predominantly between the 4th and 5th days. It differs from innate immunity in that its magnitude appears proportional to the size of inoculum used or to the number of parasites eliminated during the infection.

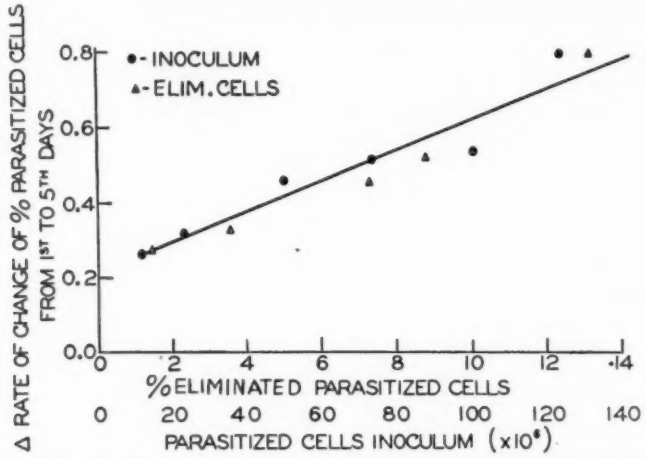

FIG. 3.—Relationship of increased size of inoculum and percent eliminated parasitized cells to the rate of change of percent parasitized cells.

Note that more parasitized cells are eliminated as the size of the inoculum increases and that this relationship appears consistent during the first 4 days of the patent infection.

To ascertain the exact number of dead parasites and the period required to produce an immune effect is not possible with the data available, inasmuch as the merozoite mean per segmenter and the length of the asexual cycle were not considered. Since during the first 4 days the rate of change of parasitemia is independent of the size of the inoculum, the number of parasites eliminated by innate immunity is proportional to the inoculating dose. An approximation of the number of parasites which had been eliminated by the 4th day was calculated by adding up the daily differences between the percent of parasitized cells estimated by using the rate for the beginning of a day (see fig. 1) and that estimated by using the average rate for that day. Such figures appear in the last column of table 2. The difference between the average logarithmic rates of increase in parasitemia on the 1st and the 5th days (table 1) may be used as an approximate measure of the immune response. It may be seen from the line in figure 3 that the magnitude of the acquired immunity varies directly with the number of parasites eliminated during the previous 4 days. Since the difference in rate between the 1st and the 4th day was practically the same (table 1) for all inoculating doses, acquired immunity did not play an important role before the 5th day. This is

TABLE 3.—*The suppressive effect of various drugs on the parasitemia of* P. lophurae.

Percent drug-diet concentration	Number of tests	Number of birds	Mean suppression of parasitemia, percent	Suppressive dose 50 ± S.E. (SD_{50}), percent	Quinine equivalent ± S.E.
			Quinine		
0.01	8	64	3.2	0.035 ±0.002 (5.19)	1.00
0.015	1	8	8.0		
0.02	10	80	24.5		
0.03	8	64	40.7		
0.04	10	80	58.5		
0.06	6	48	82.0		
			Quinacrine		
0.01	4	32	13.5	0.022 ±0.002 (9.38)	1.55± 0.16
0.015	2	16	21.5		
0.02	4	32	49.7		
0.03	3	24	67.8		
0.04	3	24	75.0		
0.06	1	8	90.0		
0.08	1	8	92.0		
			Pamaquine		
0.0005	5	40	10.2	0.0012±0.0001 (7.63)	29.40± 2.80
0.001	7	56	31.0		
0.0015	4	32	61.6		
0.002	4	32	79.7		
0.003	2	16	93.0		
0.004	1	8	94.0		
0.006	1	8	96.0		
			Pentaquine		
0.000065	2	16	6.0	0.0002±0.00004 (15.98)	158.45±26.65
0.000125	3	24	33.0		
0.00025	3	24	56.5		
0.00050	2	16	88.0		
0.001	1	8	95.0		

one reason for our choice of the 4th day of infection for the assay of antimalarials. Thus, any effect will be due primarily to the drug.

Practically identical observations have been reported by Gingrich and Schoch[6] with *Plasmodium cathemerium* in the canary. An analysis of their data may reveal relationships similar to those described in the present paper.

Assay of antimalarials.—Complete data showing the suppressive effect of quinine, quinacrine, pamaquine and pentaquine on the parasitemia are given

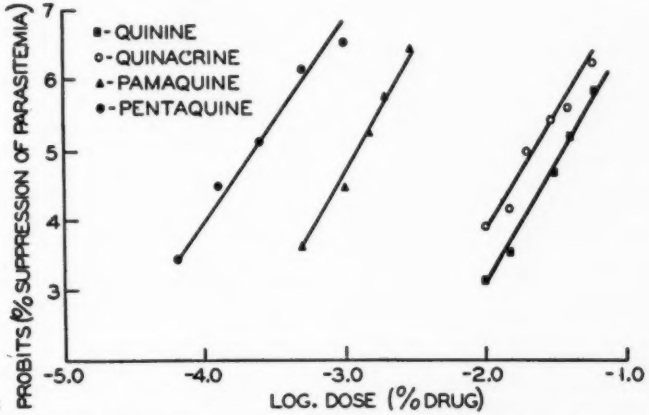

FIG. 4.—Suppression of the parasitemia by varied concentrations of quinine, quinacrine, pentaquine and pamaquine in the diet. From the linear relationship the concentration of the drug in the diet necessary to suppress the parasitemia to 50% of the control group (SD_{50}) was interpolated at probit 5.

in table 3. The relationship between the percent suppression of parasitemia and dose of drug is shown in figure 4 in terms of probits and log dose. From the straight line, the 50% suppressive dose was determined and its standard error calculated graphically by the method of Miller and Tainter.[9] As may be seen from table 3, quinine was found to have an $SD_{50} \pm$ S. E. of $0.03 \pm 0.002\%$ in the diet. Quinacrine, pamaquine and pentaquine had SD_{50} values of $0.022 \pm 0.002\%$, $0.0012 \pm 0.0001\%$ and $0.0002 \pm 0.00004\%$, respectively. These values give quinine equivalents for the three drugs of 1.5 ± 0.16, 29.4 ± 2.8 and 158.5 ± 26.7, respectively. From these data it may be seen that the quinine equivalent of antimalarials may be precisely determined in lophurae infections.

9. Miller, L. C. and Tainter, M. L. 1944, Proc. Soc. Exper. Biol. & Med. **57**: 261–264.

SUMMARY

A method is described for the evaluation of antimalarials with *Plasmodium lophurae* in the chick using a drug-diet procedure.

The concentration of drug in the diet which suppresses parasitemia by 50% is used to compare the relative potencies of antimalarials. By taking quinine as a standard, the quinine equivalents of quinacrine, pamaquine and pentaquine were found to be 1.5 ± 0.16, 29.4 ± 2.8 and 158.5 ± 26.7, respectively.

Innate immunity and acquired immunity during the course of infection are discussed. The latter differs from the former in that it develops largely after the 4th day of infection and its magnitude depends upon the number of parasites previously eliminated during the infection.

STUDIES OF BRUCELLOSIS IN UTAH

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Brucellosis is recognized as one of the most important diseases of animals transmissible to man. Its incidence in man is contingent upon the extent of infection in animals, the species of animals infected, and the species of brucella with which these animal reservoirs are infected. The extent of human infection, therefore, varies in different sections of the country depending on the type of animal industry pursued. For example,[1] from 1930 to 1943 the annual rate per 100,000 population in the major swine- and cattle-raising areas of the west north central and west south central states was 3.76 and 3.34, respectively, while in the mountain states where the animal industry consists chiefly of cattle and sheep, the annual rate was 1.99 per 100,000 population.

From February, 1947 to February, 1948, a study of human brucellosis in Utah was made in an attempt to approximate the actual number of cases occurring annually and to determine the species of brucella involved. Findings in this state are probably representative of the mountain group, in which a comparatively lower rate is reported. The following tables and figures summarize the results of a survey of 25,294 serums forwarded to the Division of Laboratories, Utah State Department of Health, for routine serologic tests for syphilis. This number represents a sampling of approximately 4% of the population of the state.

MATERIALS AND METHODS

The agglutination test used was the rapid plate method (Huddleson) as described by Hitchens.[2] The antigen employed was prepared according to the technique described in the preceding reference, using a smooth strain of *Brucella abortus* obtained by the Division of Laboratories from Dr. Huddleson in 1928. Serums were screened at 1:25 dilution, and those showing at least three-plus agglutination at this dilution were titered until an end point was reached. The highest dilution demonstrating at least one-plus agglutination was considered as the titer end point. A questionnaire was forwarded to the physician submitting a positive sample to obtain an epidemiological history from the patient. It consisted chiefly of queries as to occupation, environment, use of raw or pasteurized dairy products, animal contact or contact with carcasses or meat of farm animals, history of a previous diagnosis of brucellosis, and history of previous symptoms of brucellosis. On 292 of the serums positive for brucella, an agglutination test for tularemia was also conducted. A "cross agglutination" was observed in only four.

A three-plus reaction at 1:25 dilution was considered as evidence of exposure to brucella. There is much controversy as to whether agglutination at this low titer is the result of normal agglutinins or actual exposure to brucella. However, it is also recognized that many cases of chronic brucellosis may be characterized by

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1. Jordan, C. F. and Borts, I. H. 1947, Brucellosis and infection caused by three species of Brucella. Am. J. Med. **2**: 156–167.

2. Hitchens, A. P. 1945, Diagnostic Procedures and Reagents, New York, American Public Health Association.

TABLE 1.—*Titer distribution of 577 serums positive for brucella antigen at least 3+, 1:25 dilution.*

Titer*	Male		Female		Male and female	
	Number	Percent	Number	Percent	Number	Percent
1:25	38	13.9	39	12.9	77	13.3
1:50	132	48.2	149	49.2	281	48.7
1:100	49	17.9	69	22.8	118	20.5
1:200	22	8.0	33	10.9	55	9.5
1:500	25	9.1	8	2.6	33	5.7
1:1000+	8	2.9	5	1.6	13	2.3
Totals	274	100.0	303	100.0	577	100.0

* Highest dilution showing at least 1+ agglutination was considered as titer end point.

negative serology or agglutination in low titers. Therefore, persons whose serums agglutinated at this titer were also queried to ascertain its significance.

SURVEY RESULTS

The titer distribution of 577 serums found to be positive is shown in table 1. Thirty-eight percent reacted in titers of 1:100 and higher in both males and females. The incidence of high titers of 1:500 and above was somewhat greater in males.

The incidence of agglutinins in males and females and the distribution by county is shown in table 2. Of 25,294 serums tested, 2.28% were positive at 1:25 dilution and higher. The incidence of agglutinins was 2.48% in males and 2.13% in females. This corresponds closely to the male-female ratio of cases reported to the Utah State Department of Health. During 1946 and 1947, 113 cases of brucellosis were reported, of which 67 were males and 46 females. This narrow ratio between males and females is in marked contrast to that which was reported by Jordan and Borts.[1] Of 2082 brucellosis cases reported in Iowa from 1933 to 1944, 1639 occurred in males and 443 in females, a ratio of almost 4 to 1. They suggest that direct contact with animals was responsible for the high attack rate in males. Of 358 strains isolated from human cases in Iowa, 66% were *Br. suis*, 9% *Br. melitensis*, and only 25% were *Br. abortus*. In Utah few swine are raised and so far as has been determined the majority of brucellosis is due to *Br. abortus*. It is probable that this difference in male to female ratio is due to the role of raw dairy

TABLE 2.—*Incidence by county of persons whose serums contained brucella agglutinins.*

County	Total males tested	Percent positive	Total females tested	Percent positive	Total males and females tested	Percent positive
Beaver	29	6.90	66	0.00	95	2.11
Box Elder	253	4.35	686	2.48	939	2.98
Cache	527	3.60	1155	3.20	1682	3.32
Carbon	763	1.83	775	1.55	1538	1.69
Daggett*						
Davis	1717	1.57	515	1.36	2232	1.52
Duchesne	99	3.03	303	2.97	402	2.99
Emery	60	3.33	95	4.21	155	3.87
Garfield	51	3.92	157	2.55	208	2.88
Grand	5	0.00	18	0.00	23	0.00
Iron	161	0.62	229	1.75	390	1.28
Juab	76	0.00	129	1.55	205	0.98
Kane	12	0.00	45	0.00	57	0.00
Millard	67	1.49	191	1.57	258	1.55
Morgan	17	5.88	22	0.00	39	2.57
Piute	10	0.00	45	0.00	55	0.00
Rich*						
Salt Lake	3105	2.54	3404	1.67	6509	2.09
San Juan	21	4.76	46	0.00	67	1.49
Sanpete	148	1.35	491	1.22	639	1.25
Sevier	170	2.94	551	2.54	721	2.64
Summit	405	5.67	189	2.12	594	4.55
Tooele	387	0.78	593	2.02	980	1.53
Uintah	658	2.28	591	1.69	1249	2.00
Utah	1241	2.57	2330	2.45	3571	2.49
Wasatch	106	1.88	178	5.61	284	4.23
Washington	40	7.50	139	2.88	179	3.91
Wayne*						
Weber	911	2.85	1274	2.28	2185	2.52
Out of State	21	0.00	17	5.88	38	2.63
Totals	11,060	2.48	14,234	2.13	25,294	2.28

* No samples tested from these counties.

products infective with *Br. abortus* rather than to increased contact with animals by women in this state.

In counties in which a significant number were sampled the percent positive varied from 1.28 to 4.55. The correlation of this incidence with recognized infection in animals will be demonstrated later. In determining the source of the sample, the physician's address was used rather than the patient's. This was done, primarily, because physicians frequently omitted the patient's address on the information which accompanied serum samples, and secondly, using the patient's address would require a duplicate filing system which was prohibited by lack of personnel. In effect, this had a tendency to raise the percentage of positives in the urban counties since many rural people seek medical attention in urban areas.

The distribution of positive individuals by age and sex is shown in table 3. Of the negative samples, the age distribution was representatively determined on approximately 60%, establishing a basis of distribution for the remaining 40% whose ages were not determined. Of the positive samples, the age was known on 85%, upon which basis the unknown ages were distributed. The incidence was low in the 0 to 14 age group, moderate in the 15 to 19 group, and significantly higher in the 20 to 24 age group. This low incidence in children is in direct correlation with the number of cases observed in Iowa by Hardy and associates. It appears that, within some variation, the incidence is the same in all ages of adults between 20 and 64. Above 65 years of age the incidence drops.

The data obtained from questionnaires returned by physicians are summarized in figures 2 to 4. Of 577 questionnaires forwarded to practicing physicians, 323 were returned with information sufficient to be of some value. Some discretion must be employed in interpreting these data. The majority of these samples were obtained from apparently healthy individuals during routine examinations such as premarital, pre-natal, and pre-employment physical examinations. Because of the routine nature, an accurate history is often not obtained at such times. Also, the physician had to rely on the patient's memory, which frequently is not accurate. The percent noted in figures 1 to 4 is a percentage of each group falling in various titers.

A comparison of serum titers of positive individuals living in urban and rural areas is made in figure 1. The percentage in low titers of 1:25 and 1:50 is consistently higher in the urban group than in the rural. Approximately 71% of the urban group was positive at 1:50 and lower, while 58% of the rural group was positive at these low

TABLE 3.—*Age and sex distribution of persons whose serums contain brucella agglutinins.*

Age group	Total males tested	Percent positive	Total females tested	Percent positive	Total males and females tested	Percent positive
0–14	150	0.00	147	0.68	297	0.34
15–19	1328	1.96	2949	1.53	4277	1.66
20–24	3475	2.62	4242	2.19	7717	2.38
25–29	2048	3.08	2806	2.46	4854	2.72
30–34	1209	3.23	1693	1.71	2902	2.34
35–39	805	1.24	1039	2.79	1844	2.11
40–44	579	2.76	416	4.33	995	3.42
45–49	394	2.03	270	1.85	664	1.96
50–54	408	2.70	214	1.87	622	2.41
55–59	242	1.65	135	0.74	377	1.33
60–64	199	2.01	163	4.29	362	3.04
65–69	117	0.85	52	1.92	169	1.18
≧70	106	0.94	108	0.93	214	0.93
Totals	11,060	2.48	14,234	2.13	25,294	2.28

titers. If the brucella agglutinins found in the positive serums of this survey were due solely to infection with brucella, one would expect to find a comparable serum titer distribution in both rural and urban groups. The predominance of positive "low-titer" serums in the urban group suggests either an agglutinin response to minimal exposure such as could be acquired through ingestion of improperly pasteurized milk, an agglutinin response to the ingestion of killed brucella organisms in pasteurized milk, or a non-specific agglutination at these low titers.

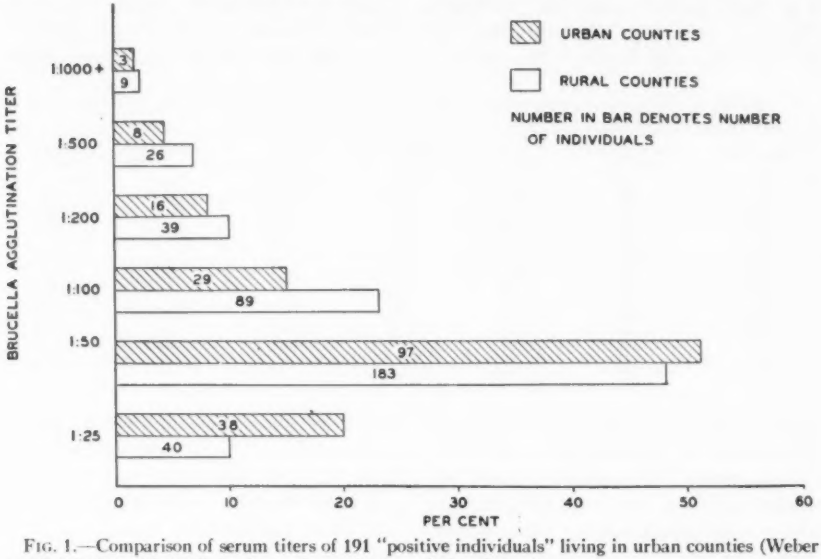

FIG. 1.—Comparison of serum titers of 191 "positive individuals" living in urban counties (Weber and Salt Lake) with 386 living in rural counties (remainder of state).

Disregarding animal contact, the effect of the use of raw dairy products versus pasteurized dairy products is demonstrated in figure 2. Of males who used pasteurized dairy products, 30% reacted in titers of 1:100 and higher, while in the group that used raw dairy products, 44% reacted in higher titers. Of females who used pasteurized dairy products, 27% reacted in higher titers, while in the group that used raw dairy products the percentage was the same as that of the corresponding male group. This difference is probably greater than is demonstrated, because people who profess to have used only pasteurized dairy products frequently forget raw milk or butter consumed during rural visits. Hence, some of those included in the group that used pasteurized products should have been included in the group that used raw dairy products. Inasmuch as there are no data available as to the use of raw or pasteurized dairy products by the negatives in the survey, it was impossible to determine whether the incidence of brucella agglutinins was higher among raw milk users.

The effect of animal contact (carcasses and meat included) and the use of raw dairy products on the serum titer of exposed individuals is shown in figure 3. Of males who had animal contact and/or used raw milk, 46% reacted in titers of 1:100 or higher, while in

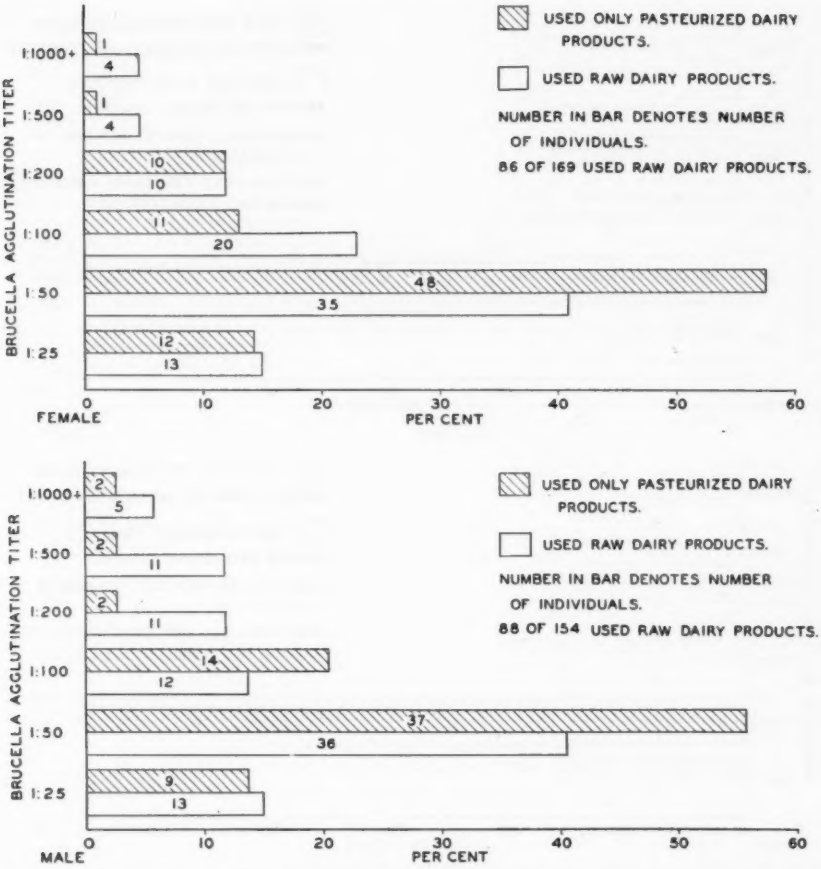

FIG. 2.—Comparison of serum titers of "positive individuals" who used raw dairy products with those who used only pasteurized dairy products, based on 323 questionnaires returned by physicians.

males who used only pasteurized dairy products and had no animal contact, 23% reacted in higher titers. Among females this relationship between the two groups closely parallels that among males. Note the absence of extremely high titers in both males and females who used only pasteurized dairy products and who had no animal contact.

In using the agglutination test as an aid in diagnosing clinical brucellosis in man, the question as to which titer is to be considered as positive often arises. Evans[3] states that a positive agglutination at a titer of 1:40 is suggestive of infection. Huddleson[4] refers to a titer of 1:50 or higher as presumptive if the patient is showing clinical evidence of

3. Evans, A. C., Robinson, F. H. and Baumgartner, L. 1938, Studies on chronic brucellosis. Pub. Health Rep. **53**: 1507–1525.
4. Huddleson, I. F. 1943, Brucellosis in Man and Animals, New York, The Commonwealth Fund.

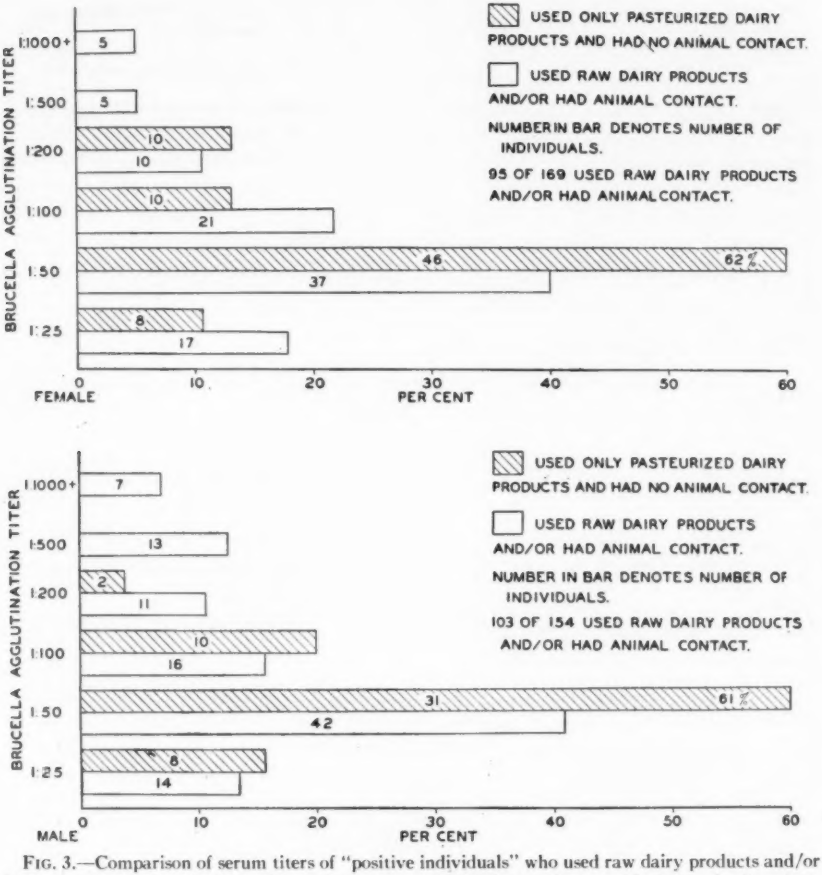

FIG. 3.—Comparison of serum titers of "positive individuals" who used raw dairy products and/or had animal contact with those who used only pasteurized dairy products and had no animal contact. Information based on 323 questionnaires returned by physicians.

infection. In this survey the relation of titer to previous symptoms is shown in figure 4. The information regarding previous symptoms was taken from questionnaires returned by physicians. Queries regarding symptoms included a history of malaise, recurrent headaches, afternoon fevers, and other symptoms commonly found in brucellosis. One is impressed by the fact that the percentage distribution in various titers is practically the same in both the symptomatic and asymptomatic groups. Hence, one would conclude that a diagnosis of clinical brucellosis could not be made accurately on the basis of a positive agglutination reaction alone. Perhaps in areas where the more virulent *melitensis* and *suis* species are prevalent, a positive agglutination titer would have more significance.

The percentage of males with a history suggestive of clinical brucellosis was 43.5, compared to 34.3% of the females giving a history of previous symptoms. These symptoms cannot be

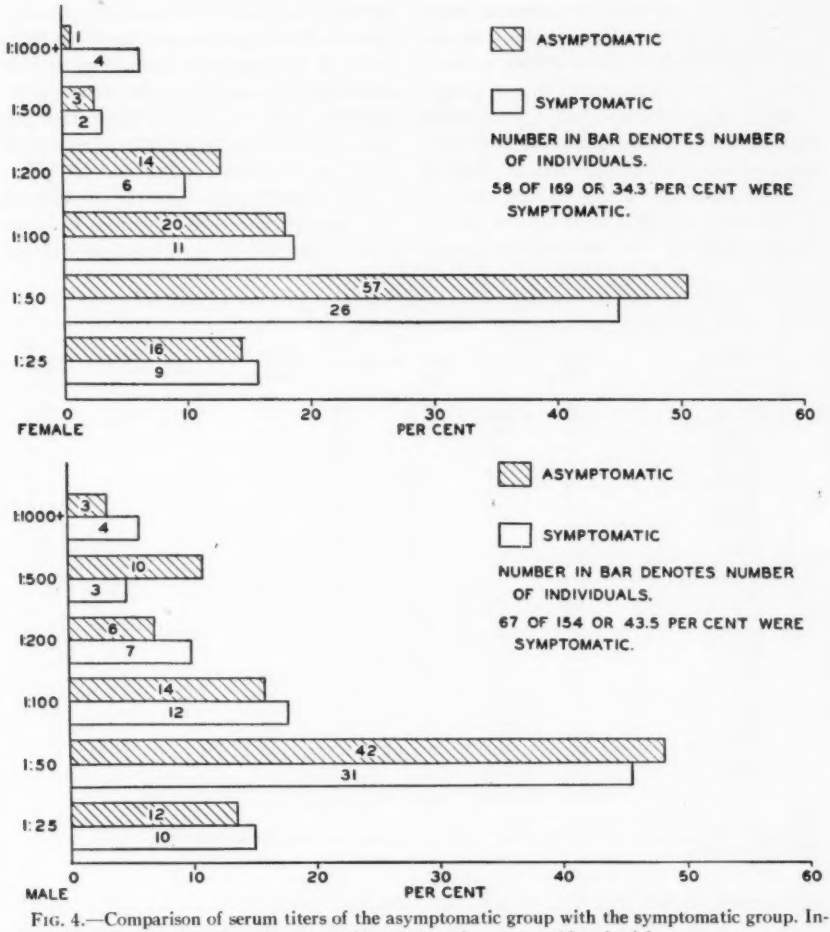

FIG. 4.—Comparison of serum titers of the asymptomatic group with the symptomatic group. Information regarding symptoms based on 323 questionnaires returned by physicians.

attributed wholly to brucellosis, as they are common symptoms in other diseases. It is interesting to note that more males than females were symptomatic. This may account for part of the higher reported incidence in males.

Based on the findings of this survey, one may roughly calculate the number of brucellosis cases annually occurring in Utah. Assuming that within reasonable error, the persons included in this survey were representative of the general population of the state, the incidence of agglutinins would be, conservatively, 2%. The length of time that agglutinins persist in the serum of a positive individual, such as would be detected through a survey, is unknown. Dooley[5] found that in a group of 15 boys who acquired agglutinins at a titer of 1:320 and higher from the ingestion

5. Dooley, P. 1932, Undulant fever. Arch. Int. Med. 50: 373.

of milk infected with *Br. abortus*, most of them had a negative serology or agglutination in low titers within a year. Only 2 of the 15 manifested any symptoms. It is also known that in some cases of chronic brucellosis, agglutinins persist for years. Assuming that one-half of of the positives acquired agglutinins within the year, the annual rate would be one-half of the percent positives sentative of the actual amount of infection as these figures are based on herd tests of farmers who are interested in controlling brucellosis. It is thought, however, that it reflects somewhat the actual incidence since the amount of infection in tested herds will be influenced to some extent by the amount of infection in surrounding herds. In some of the counties the number tested is too

TABLE 4.—*Incidence by county of bovine brucellosis in Utah during 1946 and 1947.**

County	Herds tested	Cattle tested	Reactors	Suspects	Reactors and suspects	Percent reactors	Percent suspects	Percent reactors and suspects
Beaver	288	3578	210	184	394	5.9	5.1	11.0
Box Elder	1194	8173	334	556	890	4.1	6.8	10.9
Cache	522	4657	160	774	934	3.4	16.6	20.0
Carbon	88	364	17	12	29	4.7	3.2	7.9
Daggett	6	32	1	3	4	3.1	9.4	12.5
Davis	307	1762	160	152	312	9.1	8.6	17.7
Duchesne	95	1137	66	47	113	5.8	4.1	9.9
Emery	65	571	15	10	25	2.6	1.8	4.4
Garfield	5	273	41	24	65	15.0	8.8	23.8
Grand†								
Iron	6	29	0	0	0	0.0	0.0	0.0
Juab	161	732	48	37	85	6.6	5.0	11.6
Kane	4	50	0	1	1	0.0	2.0	2.0
Millard	279	1769	102	64	166	5.8	3.6	9.4
Morgan	79	689	89	88	177	12.9	12.7	25.6
Piute	233	3172	40	55	95	1.3	1.7	3.0
Rich	204	1352	143	175	318	10.6	12.9	23.5
Salt Lake	499	2622	223	144	367	8.5	5.5	14.0
San Juan	6	1079	43	24	67	4.0	2.2	6.2
Sanpete	288	2761	160	124	284	5.8	4.5	10.3
Sevier	108	1880	206	121	327	11.0	6.4	17.4
Summit	327	3393	151	281	432	4.4	8.3	12.7
Tooele	40	496	5	21	26	1.0	4.2	5.2
Uintah	368	2195	111	130	241	5.1	5.9	11.0
Utah	421	3825	242	254	496	6.3	6.6	12.9
Wasatch	98	863	81	75	156	9.4	8.7	18.1
Washington	160	1412	113	52	165	8.0	3.7	11.7
Wayne	115	1200	45	61	106	3.8	5.0	8.8
Weber	405	3367	203	272	475	6.0	8.1	14.1
Totals	6371	53,433	3009	3741	6750	5.6	7.0	12.6

* Based on official releases of the Federal-State Cooperative Brucellosis Control Program in Utah.
† No cattle tested.

determined in the survey, or 1%, or 6500. Of this number, one-third, conservatively, (43.5% of the males and 34.3% of the females) demonstrated symptoms at some time. Hence, 2166 Utah residents would be annually afflicted with brucellosis. Compared with the 82 cases reported to the Utah State Department of Health during 1947, a ratio of 26 probable cases to each reported case is indicated.

The incidence of brucellosis in cattle in the various counties of the state is shown in table 4. The incidence noted in each county is not necessarily repre- small to sample the county adequately.

Figure 5 shows the correlation between the incidence in cattle and man according to county. Although the epidemiological studies of this survey are incomplete, this map was plotted on the assumption that most cases of human brucellosis in this state are contracted from infected cattle. In computing the rate of infection in cattle both reactors and suspects must be included as of epidemiological significance. It is true that cattle with higher titers (reactors) are more prone to disseminate brucella organisms in mammary secretions and

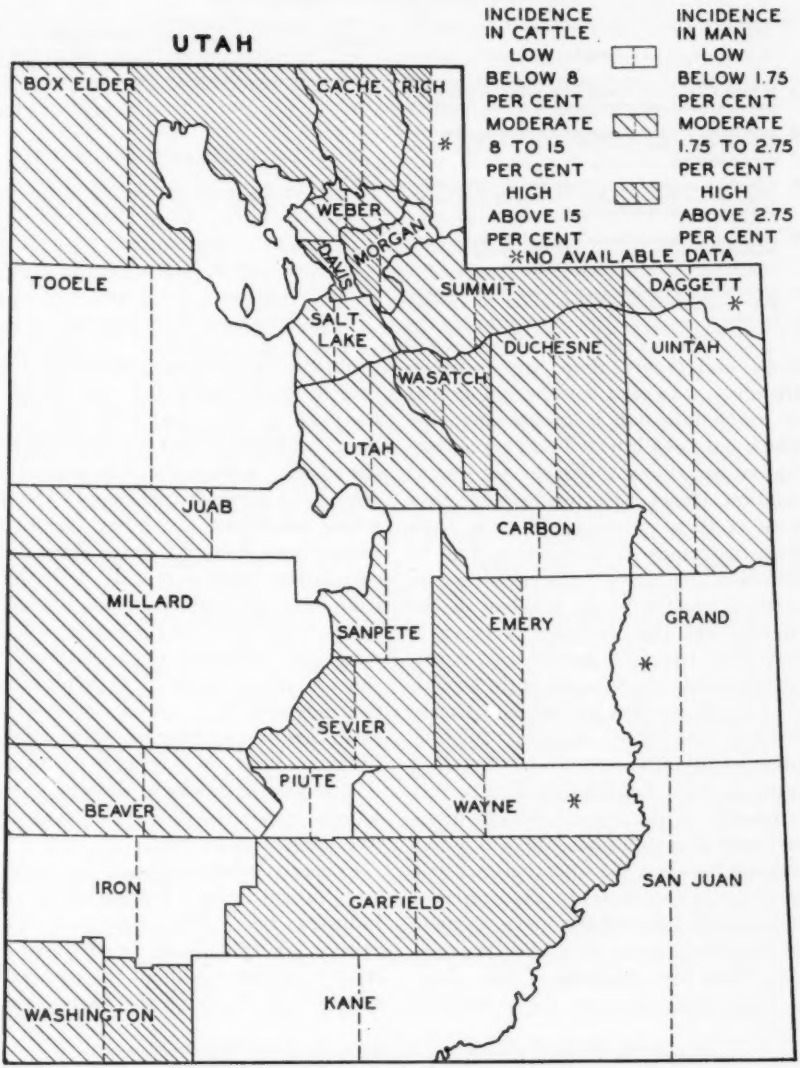

FIG. 5.—Correlation between the incidence of brucellosis in cattle and brucella agglutinins in man. Incidence in cattle based on both reactors and suspects. Data taken from tables 2 and 4.

The right and left halves of Emery County are reversed.

TABLE 5.—*Epidemiological findings of investigations made of 54 individuals whose serums were positive for brucella antigen.*

Group	Number in group	Titer of positive individuals					
		1:25	1:50	1:100	1:200	1:500	1:1000+
A. Individuals who had contact with and/or used raw dairy products from herds currently affected with brucellosis.	24	3	6	4	4	2	5
B. Individuals who had contact with and/or used raw dairy products from herds currently free of brucellosis but previously infected.	9	0	7	0	2	0	0
C. Individuals who had contact with and/or used raw dairy products from herds currently free of brucellosis and with no history of previous infection.	13	1	7	3	1	1	0
D. Individuals who had contact with and/or used raw dairy products but source not available for testing (herd sale, slaughter, etc.).	8	1	1	5	1	0	0
E. Individuals belonging to groups A and B who used raw dairy products from infected herds but had no animal contact.	16	3	6	1	3	2	1

body excrements than are suspects. However, a suspect is either an animal in which infection is progressing, one which has been infected and is recovering, or one showing non-specific agglutination. Hence, one must assume that some of these suspects will be or have been instrumental in exposing man to infection. In figure 5 the incidence in cattle is plotted in the left half of the county and the incidence in man is plotted in the right half of each corresponding county. As noted in the legend, the rate of infection has been arbitrarily divided into three degrees. In four counties no data are available on either cattle or man. Considering such factors as relative urban and rural populations and animal populations, there is a correlation between the infection in cattle and man in this State. In 14 counties of 25 on which data on both man and cattle are available there is a close correlation. With the exception of two, there is some correlation in the remaining counties. Davis County reports a high rate in cattle and a low rate in man; however, in this county the figure on human infection is not representative of the county. A majority of these samples originated in the Naval Supply Depot, whose employees are living chiefly in urban areas. Emery County showed no correlation whatsoever. It has third from the lowest rate of bovine brucellosis among counties in the State, while in man the incidence of agglutinins is third from the highest. It is probable that infected farm animals other than cattle are responsible for these agglutinins in man. A thorough investigation should be made to determine the source responsible for this comparatively high rate in man.

Concerted efforts through the cooperation of the Federal Bureau of Animal Industry, State Department of Agricultu[illegible] and the State Department of Health, should be made to eliminate animal brucellosis in the State. After

TABLE 6.—*Incidence of brucellosis in cattle tested during the epidemiological investigations of 54 positive individuals noted in table 5.*

Herds* tested	Cattle tested	Reactors	Suspects	Reactors and suspects	Percent reactors	Percent suspects	Percent reactors and suspects	Herds infected	Herd negative
47	319	57	61	118	17.9	19.1	37	25	22

* One herd of 42 cows which contained 14 reactors and 21 suspects was vaccinated as adults following an "abortion storm." This consequently raises the percentage of reactors and suspects. However, a virulent strain of *Br. abortus* was isolated from quarter milk samples of 3 of 4 reactors which were cultured. Hence, at least part of the agglutinins in this herd can be attributed to infection with a virulent strain other than strain 19.

brucellosis has been eradicated, or materially reduced for two to three years, a survey of this type should be repeated to demonstrate the effect of an eradication program on the incidence of agglutinins in man.

EPIDEMIOLOGICAL STUDIES

The epidemiological findings of investigations made of 54 "positive individuals" are summarized in tables 5 and 6. At the beginning of the survey, it was fully intended to investigate all cases whose returned questionnaires indicated animal contact or use of raw dairy products. However, these investigations were curtailed because of a more urgent assignment. The data obtained from this small number of cases cannot be considered as entirely representative of the epidemiology of human brucellosis in Utah.

Of the 54 cases investigated, 8 indicated contact with swine, 6 with sheep, 14 with horses, 25 with cattle, and none with goats. In this survey only the most plausible source was tested because of limitation of personnel and time and a not infrequent lack of cooperation and interest on the part of the farmer. Most of the persons indicating contact with swine had such contact while raising a hog or two for farm butchering. On two occasions it was indicated that swine were the probable source of infection. However, in both instances neither the permission to test nor the assistance of the farmer could be obtained.

In 24 of the 54 cases investigated, human infection could be traced to animals which were currently infected. In 9 cases, herds were currently free of infection but brucellosis had been previously recognized in their herds. In group A, persons used raw milk or had contact with herds which were currently infected. Fifteen of the group had titers of 1:100 and higher, while only 9 had titers of 1:50 and lower. This ratio is reversed in groups B and C, which had contact with or used raw milk from herds which were currently free of brucellosis. Group E includes those individuals of groups A and B who acquired agglutinins through the ingestion of raw dairy products. This consisted of almost 50% of the number in groups A and B. In the remainder of groups A and B it is difficult to determine by which means infection was acquired, as they had used raw milk and also had animal contact.

During the course of these investigations, 47 herds were tested. Twenty-five were found to be infected. On two occasions, an individual had contact and used raw dairy products from two infected herds. On one occasion, two individuals reported the use of raw milk from the same herd. This herd consisted of 32 animals, of which 12 were reactors and 4 were suspects. Two recognized cases of brucellosis were reported to have used milk from this dairy. The local health department investigated, determined that the herd was infected, and immediately prohibited the further sale of raw milk from this herd. This episode occurred prior to July, 1947, after which no raw milk could legally be sold through regular trade channels in this State. Of the two persons detected through the survey, one had a titer of 1:500 and gave a negative history of any symptoms, while the other had a titer of 1:1000 and had been hospitalized in December, 1946 with a fever of unknown origin which was accompanied by an acute pneumonitis.

Fifteen quarter milk samples were taken from 4 reactors of this herd. *Br. abortus* was isolated from 12 of the 15 samples. Cream streaks on tryptose agar plates from two quarter samples were so heavily seeded with brucella colonies that it was possible only to estimate the number of colonies. If the remainder of the infected animals were shedding brucella at a comparable rate,

this dairyman was certainly delivering a massive dose of *Br. abortus* with each bottle of milk.

Isolation studies were conducted on quarter milk samples in accordance with recognized procedures. Strains isolated were identified as being brucella by colonial characteristics, Gram stain, and agglutination studies. Strains were typed, using basic fuchsin and thionin dyes, the former in concentrations of 1:25,000, 1:50,000 and 1:75,000, and the latter in concentrations of 1:50,000 and 1:75,000. Typing of isolated strains was confirmed by Dr. Wayne Binns, Utah State Agricultural College, Logan, Utah.

A total of 164 quarter samples from 41 cows were cultured. With the exception of 4 cows, duplicate quarter milk samples were forwarded to Dr. Binns, who kindly cooperated in conducting isolation studies by guinea pig inoculation. *Br. abortus* was isolated by cultural methods from 46 quarters, representing isolations from 17 cows. By guinea pig inoculation, *Br. abortus* was isolated from 10 of the 37 cows. Using both methods, isolations were made from 19 cows. Eight cows were positive by both methods. Two were positive only by animal inoculation and 5 were positive only by cultural method. (This does not include isolations from 4 cows on which only the cultural method was used.) All strains were typed as *Br. abortus*.

Whey agglutination tests were conducted on quarter samples. The relation of the whey agglutination titer to the presence of brucella in the milk of reactors is demonstrated in the following: of 45 negative samples, no isolations; 21 positive at 1:25 dilution, 2 isolations; 23 positive at 1:50, 1 isolation; 11 positive at 1:100, 1 isolation; 21 positive at 1:200, 10 isolations; 43 positive at 1:500 and higher, 32 isolations.

Although brucellosis is perhaps recognized as one of the most important diseases of animals transmissible to man, its actual significance as a public health problem is not fully appreciated. Its recognition in this state is conservatively calculated to be 1/26 of its actual incidence. The basic attack to the public health problem of human brucellosis is the removal of infection from animal reservoirs. To attain this goal, concerted action of public health agencies and the agencies directly responsible for its control in animals will be necessary. Until this goal is reached, we must also resort to adjunctive control measures such as pasteurization of dairy products and educational programs directed at rural populations and occupational groups which are constantly exposed to this disease.

SUMMARY AND CONCLUSIONS

1. In a survey of 25,294 individuals, the incidence of agglutinins for *Brucella abortus* was 2.28%.

2. The incidence of agglutinins in serums was 2.48% in males and 2.13% in females.

3. Children presented the lowest incidence of agglutinins and, within limits, the incidence in adults was equal in all age groups.

4. The effect of animal contact and the use of raw milk on serum titers of exposed individuals was demonstrated.

5. Of 323 positive individuals, 43.5% of the males and 34.3% of the females gave a history of symptoms suggestive of brucellosis.

6. The actual number of cases occurring annually is estimated to be 26 times the number reported.

7. The incidence of agglutinins in man is correlated with the amount of infection in cattle.

8. Epidemiological investigations suggest that *Br. abortus* is the chief cause of human brucellosis in Utah.

AN ATTEMPT TO CONFIRM AN "IN VITRO CULTIVATION OF RAT LEPROSY ORGANISM"

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A recent publication by Ashbel and Poljakoff[1] indicated that acid-fast bacteria can be cultivated from rat lepromata after incubation for a few weeks to 1½ years and can be shown to cause rat leprosy following reinoculation into rats. In subculture these organisms grow rapidly (1 to 3 days) and produce smooth, grey- to orange-colored colonies. In 1947 Dr. Ashbel demonstrated to us sections of lesions which were said to have resulted from the inoculation of such cultured organisms. Since these lesions were unquestionably rat lepromata, an attempt has been made with the assistance of Dr. Ashbel to confirm the reported findings. It is the purpose of this communication to summarize and discuss the results obtained.

METHODS

With the assistance and supervision of Dr. Ashbel, two types of confirmatory experiments were initiated: (a) the cultivation of acid-fast bacteria from rat lepromata and (b) the production of rat leprosy by inoculation of the cultivable acid-fast bacilli provided by Dr. Ashbel. The methods were those of Ashbel and Poljakoff, with the following exceptions: (a) the bacillary suspensions for the inoculation of culture medium were derived from nonulcerated lepromata, (b) three of the four groups of 20 rats receiving cultivated organisms were inoculated with pure cultures, and (c) these rats were housed in quarters in which rat leprosy had not previously been studied. A group of 50 control rats included 20 inoculated with an autoclaved suspension of Ashbel culture #457 and 30 animals which were held as unmodified controls to measure the incidence of spontaneous disease or tumors.

The culture mediums were of three types: Loewenstein's egg medium colored with congo red, Noguchi's leptospiral medium (semisolid agar with 10% rabbit serum), and Kligler's modification of the same, employing 10% rabbit blood. The Wistar rats came from a subfamily in which susceptibility to rat leprosy had been increased by selection and inbreeding. They were approximately 4 months of age at the time of inoculation.

RESULTS

Cultivation experiments.—Aside from a 3.3% incidence of contamination, the cultivation results after one year (see table 1) were negative. There was no colonial growth or other evidence that the acid-fast bacilli had multiplied. Microscopic studies at the end of 6 months revealed that even in tubes where moisture was retained the rat leprosy bacilli had declined in numbers and deteriorated in quality.

In the intervals between replacements of water the medium in cotton-stoppered tubes became dry, hard, and cornified. As may be noted below, this circumstance appears to be unfavorable even for the types of acid-fast microorganisms cultured by Ashbel and Poljakoff.

Stock cultures for the inoculation of rats.—Dr. Ashbel received from Jerusalem 18 stock cultures of acid-fast bacteria in semisolid agar medium which had become dehydrated after the fashion described by Ashbel and Poljakoff as appropriate for the cultivation of the causative agent of rat leprosy. These cultures had been derived from biopsies from 6 different rats. One of these stock

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We are indebted to Dr. J. H. Mueller for arrangements which made this study possible and for review of the manuscript.

1. Ashbel, R. and Poljakoff, A. 1948, In vitro cultivation of the rat leprosy organism. J. Infect. Dis. **83**: 279–282.

cultures, #756, contained acid-fast facilli in pure culture. Two others, #457 and #500, proved to be viable, and it was possible to separate them from the accompanying contamination. From the remaining 15 tubes it was not possible to obtain colonies of acid-fast bacteria by methods and mediums used in work on pathogenic mycobacteria or by Gordon and Hagan's[2] procedures for the isolation of acid-fast bacteria from soil. It appears probable that these 15 cultures were nonviable when received. The contaminants present in these cultures included at least two species of fungi and one of *Actinomyces*, gram-positive spore-bearing rods, gram-negative encapsulated bacilli, and assorted gram-positive cocci.

TABLE 1.—*Results in bacteriological medium inoculated with 10% emulsions of rat lepromata.*

Number of nodules	Tubes of medium inoculated	Loewenstein's medium					Noguchi's medium			Kligler's medium		
		37 C		Room temperature			Room temperature			Room temperature		
5	240*	30†	30‡	30†	30‡	30§	15†	15‡	15§	15†	15‡	15§
Positive growths:												
Acid-fast bacilli		0	0	0	0	0	0	0	0	0	0	0
Contaminants		0	0	1	2	1	1	0	0	1	1	1

* Forty-eight tubes from each nodule suspension.
† Cotton-stoppered tubes; water replaced 3 times in one year.
‡ Screw-cap tubes; water replaced 3 times in one year.
§ Sealed tubes; atmosphere replaced 3 times in one year.

The lesions produced in rats.—The routes of inoculation and the dosages employed for the infection of 4 groups of rats with the organisms of Ashbel and Poljakoff are indicated in table 2. The total bacteria injected represented 64 cc of dense suspension from actively growing cultures. One drop of India ink diluted 1:10 was added to each ml of suspension to permit identification of the sites of inoculation.

The numbers of bacilli injected were large enough to produce palpable lesions in the skin and the groin sites within one or two weeks following inoculation. Though there was no ulceration through the skin, the majority of the lesions biopsied between the 42nd and the 98th days contained necrotic centers. Spreads prepared from the firmer tissue surrounding these softened areas revealed that the preponderant phagocytic cell was the granulocyte, and that these cells were damaged by the ingestion of a modest number of bacilli.

After a few autopsies had been performed on animals suffering relatively early deaths, it became evident that the bacilli which drained to lymph nodes likewise caused a localized supperative reaction rather than the more diffuse and benign infiltration which results from the seepage of rat leprosy bacilli to these organs. Other occasional autopsies during the middle portion of the year showed that the lesions had slowly healed and that the necrotic masses had become segregated into small encapsulated "pearls" with soft or caseous centers. Stainable bacilli could at this time be found only in the caseous pearls, where they were deprived of contact with tissue cells and were slowly deteriorating into acid-fast debris. It was impossible to find any bacilli lurking in histiocytes or any small groups of epithelioid cells with rosy cytoplasm.

Just before the close of the year an epidemic of rat pneumonia began to cause scattered deaths among the animals, and the experiments were terminated in order to make final observa-

2. Gordon, R. E. and Hagan, W. A. 1937, The isolation of acid-fast bacteria from soil. Am. Rev. Tuberc. **36**: 549–552.

tions on freshly killed animals. Only those animals which were autopsied between the 10th and 12th months after inoculation are included in the present summary.

Sixty-seven of the 80 rats inoculated with live culture, 19 of the 20 receiving autoclaved culture, and 26 of the 30 unmodified controls were autopsied within this period. Twenty of the 67 autopsied rats from the infected group and 6 of the 19 which received heated culture were subjected to a careful bacteriological survey of the inoculated sites and strategically located lymphoid tissues.

Gross findings revealed that the absorption of the caseous abscesses in the skin and groin sites had progressed sufficiently so that these localities were often indicated only by the persistence of the carbon included in the inoculum. The great omentum was usually studded with the caseous pearls, some of which had become calcified. Aside from those lungs which gave evidence of pneumonic infection and the involved retrosternal and tracheobronchial lymph nodes, the organs appeared normal.

Enlargement of mammary glands occurred in 2 of the 31 females which had received live cultures, in 1 of the 10 inoculated with autoclaved culture, and in none of the 26 unmodified controls.*

The microscopic examination of lesion sites, lymph nodes, and spleens again failed to reveal acid-fast bacilli outside of the circumscribed caseous abscesses. It was again impossible in any of these materials to find collections of histiocytes or epithelioid cells with rosy cytoplasm which might suggest an incipient leprous infection.

The failure of these microorganisms to produce leprosy or other mycobacterial disease is consistent with other observations now to be summarized.

TABLE 2.—*Inoculation of rats with cultured organisms of Ashbel and Poljakoff.*

Rat group	Number of rats	Culture no.	Turbidity*	Volume of culture (cc) injected into each site				
				Skin	Groin	Peritoneal cavity	Testes	Heart
I	20 ♂	457	5	0.1†	0.1	0.5‡		
II	10 ♂			0.1	0.1 (2)‡	0.5	0.2‡	
	10 ♀	457	6	0.1	0.1 (2)	0.5		
III	10 ♂			0.1	0.1‡	0.5‡		
	10 ♀	500	3	0.1	0.1 (2)	0.5		
IV	20 ♀	756	6	0.1	0.1			0.5
C′	10 ♂	457	6	0.1	0.1 (2)	0.5	0.2	
	10 ♀	(autoclaved)		0.1	0.1 (2)	0.5		
C	30 ♀	(unmodified controls)						

* Turbidity on MacFarland nephelometer scale.
† Multiple skin sites prepared for biopsy; (2) indicates both groins.
‡ Tissues biopsied from one or more animals in attempt to recover viable microorganisms.

Fate of the bacilli in rats.—Beginning at the 42nd day after inoculation, recovery of viable acid-fast bacteria from tissues biopsied from rats in groups I, II, and IV was undertaken as follows: on the 42nd day, three groin sites and one omentum from group IV; between the 60th and 75th day, two skin sites, one large subcutaneous lesion on the abdominal wall near the site of the intraperitoneal injection and one omentum from group I; and between the 94th and 98th days, three skin sites, one groin site, and one testis from rats of groups I and II. Correct location of the sites

* We are indebted to Dr. Alwin W. Pappenheimer for the study of these tumors. Histologically these masses proved to be chronic fibromatoses associated with senile atrophic changes.

was insured by the presence of palpable or visible early lesions, by the India ink included in the inoculum, and by microscopic demonstration of the abundant acid-fast bacteria which persisted after these intervals. At least three tissue fragments from each site were placed on one or more tubes of starch agar and of Loewenstein's medium. In the case of the omenta, the large subcutaneous lesion, and the testis, 10% tissue emulsions were also spread on solid medium. Though these materials were rich in acid-fast microorganisms when biopsied and revealed abundant bacilli during the course of their incubation at 31 C for 60 and 90 days, not a single colony of acid-fast bacilli developed.

Since Loving[3] recovered Duval's chromogenic "leprosy bacilli" from inoculated rabbits after 4 and 8 months, and Hanks and Evans[4] had on several occasions recovered prompt growth of *Mycobacterium phlei* from abscesses in rabbits after 60 days, the foregoing observations suggested that the organisms of Ashbel and Poljakoff had lost their viability rather promptly after inoculation into rats. Dr. Ashbel's statement that these organisms could be cultivated from lepromata only by incubation at room temperature raised the question whether they were capable of proliferation at the body temperature of the rat.

The optimal temperature for the multiplication of the microorganisms was found to be definitely below the body temperature of the presumed host. In comparisons carried out on two types of solid mediums inoculated with approximately 0.001 mg of moist bacilli and incubated for 4 days at 31 C, the growth was rated as 4 times more luxuriant than at 36.5 C. At 38 C, the body temperature of the rat, all of the cultures were inhibited for three days. In the case of cultures #457 and #500 inoculums producing less than 100 colonies after three days at 31 C failed to grow in two weeks at 38 C in a water bath. Inoculums which produced innumerable colonies at 31 C in three days produced at 38 C an average of 9 colonies per tube in two weeks. Though heavy inoculums of culture #756 were inhibited at 38 C for three days, this strain was capable of establishing luxuriant growth during two weeks at 38 C.

3. Loving, W. L. 1941, The experimental infection of rabbits with Duval's chromogenic acid-fast bacillus from human leprosy. J. Infect. Dis. **68**: 193–206.

4. Unpublished data.

DISCUSSION

The present study provides but another example of the many failures to confirm a reported cultivation of the causative agents of human or rat leprosy, or to validate the significance of the acid-fast microorganisms which from time to time have been recovered from such lesions. The investigation affords no clues concerning the means by which leprous lesions were induced in the animals of Ashbel and Poljakoff following the inoculation of cultivable mycobacteria. It is hoped that further work by these authors may eventually throw more light on this question.

The best known agents of mycobacterial disease are the three types of tubercle bacilli and the Johne's bacillus. Each of these species when grown in vitro tends to retain growth requirements or other properties which distinguish it as a pathogenic member of the genus. It is reasonable to expect that leprosy bacilli, when successfully cultivated, will likewise possess nutrient requirements or other characteristics which will permit them to be differentiated from saprophytic species. The following observations suggest that the organisms of Ashbel and Poljakoff are

not pathogenic mycobacteria: (a) they grow rapidly on diverse and ordinary nutrient mediums, (b) they form smooth, grayish to orange butyrous colonies, and grow diffusely or disperse readily in liquid medium, (c) their optimal growth temperature is approximately 7 C below that of the presumed host, (d) they produce a suppurative type of local lesion in rats, (e) they did not remain viable as long as 42 days after injection into rats, and (f) they failed to produce rat leprosy or other mycobacterial disease.

The authors do not question the fact that mycobacteria have been cultivated from lesions of human and rat leprosy, since one of them has also recovered three strains of acid-fast bacteria from human leprous nodules.[5] The microorganisms, however, were encountered only under questionable circumstances, i.e., in two instances when ulcers existed in the patient's skin distal to the site of biopsy and in one instance from nodule fragments which required treatment with acids because of prompt growth of non-acid-fast microorganisms. Bacteriological study of these microorganisms made it necessary to conclude that they were saprophytes. Dr. Ruth Gordon at that time kindly carried out independent observations on the properties of these strains and reported[6] that they were indistinguishable from certain of the mycobacteria which Gordon and Hagan had isolated from soil. Having learned in this early work to avoid the use of materials which were open to such sources of error, a great number of lesions have since been studied by both bacteriological and tissue culture methods without again encountering cultivable mycobacteria. It seems not impossible that the organisms of Ashbel and Poljakoff may have been obtained by the use of ulcerated lesions as starting material or were introduced along with the assorted microorganisms found in their stock cultures.

5. Hanks, J. H. 1941, Behavior of leprosy bacilli in complex liquid media with highly available sources of nutrient and accessory substances. Internat. J. Leprosy, 9: 275–298.

6. 1941, Personal communication.

SUMMARY

It has not been possible to cultivate acid-fast bacteria from rat lepromas by the method of Ashbel and Poljakoff or to confirm the production of rat leprosy by inoculation of their organisms into rats.

EYELID LESION OF CHICKS IN ACUTE DIETARY DEFICIENCY RESULTING FROM BLOOD-INDUCED *PLASMODIUM LOPHURAE* INFECTION

I. DESCRIPTION; ROLE OF PANTOTHENIC ACID AND BIOTIN

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A "pellagra-like syndrome," of which granular, sticky eyelids was a part, was produced in chicks by Norris and Ringrose[1] when either dried raw egg-white or purified casein was used to supply the major protein element in the ration. The symptoms, particularly those of the eyelids, were more severe in birds receiving egg-white than in those on purified casein. The syndrome, now more simply called dermatitis, was described before the identity of the vitamin constituents of the B_2- (or G-) complex was elucidated, but it is sometimes treated in the literature under symptoms of pantothenic acid deficiency, as by Sherwood and Couch.[2] Bauernfeind et al,[3] however, point out that through the work of a number of workers, whom they name, it has been established that Norris and Ringrose were observing two etiologically distinct syndromes, one a biotin deficiency conditioned by feeding raw egg-white and the other a pantothenic acid deficiency conditioned by feeding purified casein.

Thus the present interest in the eyelid disorder to be described subsequently, which appeared during *Plasmodium lophurae* infection in chicks on a certain diet, is directed into a consideration of the literature on the relationship of pantothenic acid and biotin to sporozoal infection. Becker and Smith[4] and Becker, Manresa, and Smith[5] reported that when pantothenate was included in their ration, rats infected with the coccidium *Eimeria nieschulzi* eliminated larger numbers of oöcysts than the controls on a ration limited in respect to this vitamin. Trager[6] found that the presence of pantothenate favored the survival in vitro of *P. lophurae*, but the same author[7] also found that infected chicks and ducklings on a pantothenic acid-deficient ration experienced neither heavier nor lighter infections than those on the complete ration. A biotin-deficient regimen, however, so lowered the resistance of the birds that higher parasitemias and more deaths attributable to

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1. Norris, L. C. and Ringrose, A. T. 1930, Science, **71**: 643.
2. Sherwood, R. M. and Couch, J. R. 1948, Chapt. VII in Biester, H. E. and Schwarte, L. H. Diseases of Poultry, Ames, Iowa.
3. Bauernfeind, J. C., Norris, L. C. and Heuser, G. F. 1942. Poultry Sci. **21**: 142–146.
4. Becker, E. R. and Smith, L. 1942, Ia. State College J. Sci. **16**: 443–449.
5. Becker, E. R., Manresa, M. and Smith, L. 1943, Ia. State College J. Sci. **17**: 257–262.
6. Trager, W. 1943, J. Exper. Med. **77**: 411–420.
7. Trager, W. 1943, J. Exper. Med. **77**: 557–582.

parasitemia resulted. Bracket et al,[8] however, in a study of blood-induced infections of *Plasmodium gallinaceum* in chicks, obtained significantly higher parasitemias in birds on the pantothenate-supplemented ration than in those on the basal (deficient) ration. Seeler et al,[9] like Trager,[7] obtained higher peak parasite counts in *P. lophurae* infections in chicks on a diet deficient in biotin than in chicks on an identical ration containing adequate amounts of the vitamin.

MATERIALS AND METHODS

The problem under investigation at the time of the discovery of the eyelid lesion was the effect of diet on the course of *P. lophurae* infection in birds. The lesion appeared in 4 series of birds, but only in the group in each series on our G-ration. The formula of the ration in parts by weight is as follows: yellow corn meal, 32; gray wheat shorts, 25; ground hulled oats, 15; wheat bran, 10; alfalfa meal, 5; dried powdered buttermilk, 5; tankage, 5; limestone, 2; salt, 1; cod-liver oil concentrate, 0.25. According to the manufacturer's declaration, the cod-liver oil concentrate contains 3000 U.S.P. units of vitamin A and 400 A.O.A.C. chick units of vitamin D per gram. This strength is 4 to 5 times the U.S.P.X. standards for cod-liver oil which became official January 1, 1935. The S-ration mentioned below is identical to the G-ration in respect to the last 6 items, but the other 4 were as follows: yellow corn meal, 57; soy bean oil meal, 9; linseed oil meal, 6; gray wheat shorts, 10. Calculated total protein: G-ration, 16.14%; S-ration, 17.92%.

Microbiological assays of the biotin and pantothenic acid content of the G-ration and S-ration were made by Dr. Gladys Everson and Miss Elizabeth Wheeler of the Foods and Nutrition department. The sample was prepared for biotin assay by autoclaving with 4 *N* sulfuric acid for two hours. *Lactobacillus arabinosus* was the test microorganism. The results were 0.21 μg of biotin per gram of G-ration and 0.22 μg per gram of S-ration. The sample for pantothenic acid assay was treated with Mylase P, incubated at 37 C for 24 hours, and autoclaved. *Lactobacillus casei* was the test organism. The pantothenic acid content was found to be 20.1 μg per gram of G-ration and 18.0 μg per gram of S-ration. Thus it can be stated that the content of the two rations as regards the two vitamins was comparable. So far as biotin is concerned, however Wright et al[10] state that this vitamin is likely to be present in excess in ("unspoiled") foods because it is manufactured by microorganisms.

New Hampshire Red chicks were received from the hatchery the day after hatching, and were started on commercial rations which we had found satisfactory. After 5 days in this ration the chicks were banded, separated into groups, and started on the test rations. Calcium pantothenate supplements were mixed on the ration, but biotin in solution was administered daily directly into the crop by means of a rubber catheter attached to a syringe.

On the 12th or 14th day of life the chicks were inoculated intravenously in the wing with 1×10^8 to 2×10^8 parasitized cells from 4- or 5-day infections, in diluted citrated blood. Blood smears were stained in Giemsa. The method of counting has been described previously.[11]

RESULTS

The eyelid lesions to be described appeared in individuals of one group on the G-ration in 5 of 8 series of tests. Following are the pertinent facts regarding the appearance of the lesions: (1) They appeared in the birds on the unsupplemented G-ration infected with *P. lophurae*, (2) they did not appear in infected birds on the S-ration, (3) nor in infected birds on a mixture of equal parts of the G- and S-rations, (4) nor in infected birds on the commercial chick starter whose exact formula was ununknown to us, (5) nor in infected birds on the G-ration receiving a supplement of either 15, 6, 5, or 0.5 mg. of calcium pantothenate powder in each 100 g of the feed, (6) nor in infected birds on the

8. Brackett, S., Waletzky, E. and Baker, M. 1946, J. Parasit. **32**: 453–462.
9. Seeler, A. O., Ott, W. H. and Gundel, M. E. 1944, Proc. Soc. Exper. Biol. & Med. **55**: 107–109.
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11. Becker, E. R. 1949, Ia. State College J. Sci. **23**: 189–194.

G-ration tubed daily with 2, 1, 0.5 or 0.25 μg of biotin in 1 cc of water, (7) nor in infected birds receiving both calcium pantothenate and biotin supplements, (8) nor in uninfected birds on the unsupplemented G-ration, (9) nor in birds on the G-ration in which the parasitemia did not attain a certain intensity within 96 hours.

The only part of the dermatitis syndrome which developed in our chicks was the eyelid condition. In the most pronounced cases the first prodrome is a noticeable hypersensitivity to light, betrayed by the closing of the eyelids when exposed to strong light. The next is a slight wateriness of the eyes and tiny discolored patches on the rugose margin of the lower eyelid. The involvement of the lower lid progresses rapidly from this point, so that all or a part of it becomes soiled with moist exudate (fig. 2 and 3). Part of the exudate at least has its origin inside the eyelids, but it is likely that most of it oozes from the surface of the inflamed lower lid. The conjunctiva lining the eyelids is definitely injected in some cases, but there is no marked general puffiness of the lids, although one or more small swellings may be observed on the margin of the upper lid. There may be a slight festering of the eyelids along the lines of meeting. There is no evidence that the nictitating membrane is involved. The account so far depicts the usual developments on the 5th or 6th day of the infection.

In the severe cases the exudate on the lower lid gradually hardens so that by the end of the 8th day there is an encrustation over the lower lid and in some cases the lower margin of the upper lid (fig. 4). The tissues of the lower lid become incorporated into the encrustation, resulting in dry gangrene of the organ. The eyelids cannot open under this extreme condition, but in the milder cases they may be found festered together at one time and open later. In most of our cases the right side is more severely afflicted than the left. The scabs come off from the 9th to the 12th day. It is not certain that the scab always comes off entire, but in several cases most of it was noted either pendant from the lower margin of the upper lid or connected to the tissue bordering the lower lid (fig. 5). The eroded lower eyelid comes off with the scab (fig. 6) and a replacement, smooth and denuded of feathers, begins to regenerate from the peripheral remnant of the lid. The rugae of the margins of the upper lid disappear in the involved areas.

The healing process from this point is a progressive growth of tissue across the eye from below and behind, resulting, in the severest cases, in a complete covering for the eyeball (fig. 10). In the completely healed lesion, about three weeks after inoculation, there is little or no evidence of a seam along the line of fusion. In other cases the fusion is incomplete, so that either a small "peephole" remains at the front canthus (fig.

PLATE 1

FIG. 1.—Chick of group 4, series 5 on equal parts of G- and S-rations; 19th day of infection; eye normal (note rugose margins of the eyelids).

FIG. 2.—Chick of group 1, series 7 on G-ration; 5th day; lower lid of left eye soiled and moist with exudate.

FIG. 3.—Same chick as in figure 2; 5th day; right eye closed from hypersensitivity to light; lid more seriously affected than left lid.

FIG. 4.—Chick of group 2, series 5 on G-ration; 6th day; scab covers both lids of the eyes.

FIG. 5.—Same chick as in figure 2; 10th day; mummified right lower lid ("scab") detached except at bottom.

FIG. 6.—Same chick as in figure 5; 10th day; shortly after loss of the mummified lower lid.

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8, 9, and 11) or, in the milder cases, an opening, more or less half the size of the normal. The margin of the opening is devoid of rugosities.

The less severe form of the lesion usually results in a prominent notch in the lower lid (fig. 7) which in time becomes less prominent as it partially fills in with regenerating tissue. The rugae of the lid remain normal except in the region of the notch. The notch results from the loss of a smaller scab than what develops in the severe cases described above.

The circumstances under which the lesion appears will next be discussed, and frequent reference will be made to the data appearing in table 1. In every positive case the afflicted bird had been consuming unsupplemented G-ration at the time of inoculation. In no case did the lesion develop in birds on S-ration (series 1, 2, 3, 4, and 5), which is rather surprising because of the practically equal biotin and pantothenic acid content of the two rations. Neither did the lesions develop in 5 chicks fed a mixture of equal parts G-ration and S-ration. Chicks which were tubed with biotin in amounts of 2, 1, 0.5, or 0.25 μg daily did not develop the lesion, nor did chicks receiving 15, 6, 5, or 0.5 mg of calcium pantothenate per 100 g of the ration.

The table records a number of groups on the G-ration in which the affliction did not develop (series 4, 6 and 8). In these cases 4th-day counts did not reach the peaks attained in the series in which there was development. Series 4 was inoculated with the same inoculum as series 3, except that the injections were made about two hours later. During this time the parasites lost in vitality, and their reproductive potential was lowered, as a comparison of the 4th- and 5th-day counts for series 3 and 4 would indicate. In series 6 no lesions developed, which is not surprising in view of the 4th- and 5th-day counts, which are by no means comparable to those in series where there were lesions. The chicks of series 8 were of the same hatching as those of series 7, and the number of parasites inoculated was the same, but the two series were inoculated from different passage chicks. These two passage chicks were of the same hatching. The percent of parasitized cells was about the same in the two chicks (70% and 74%), and the dilutions of blood used for the inoculum were not very different (1:5 and 1:5.4). Despite these similar circumstances the mean 4th-day count for group 1 of series 7 was 70% and for group 1 of series 8 was 43%, and the lesions appeared in half the birds in the former but in none of the latter. The lesions that appeared were pronounced, but it should be pointed out that in these two series the age of the chicks was 14 days, whereas 12-day birds were used in the other series; and the microorganism was at the end of the 5th day when passed, whereas in the other series it was at the end of the 4th day.

DISCUSSION

The eyelid affliction which has been

PLATE 2

FIG. 7.—Same chick as in figure 2; 10th day; prominent notch in margin of lower lid, effect of the less severe form of the affliction.

FIG. 8.—Same chick as in figure 4; 19th day; neoplastic covering has replaced lost lower lid and damaged part of upper lid of left eye.

FIG. 9.—Same chick as in figure 8; 42nd day; left eye.

FIG. 10.—Same chick as in figure 9; 42nd day of infection; neoplasm completely covers right eye.

FIG. 11.—Chick of group 2, series 1 on G-ration; 90th day of infection; healing process apparently completed.

described undoubtedly has much in common etiologically and pathologically with the similar conditions observed by Norris and Ringrose,[1] but is unique in the suddenness of the onset, its acuteness and severity, and the nature of the neoplasm replacing the lower eyelid. It is likewise unique etiologically, for it develops in chicks consuming a ration that would ordinarily be accepted as adequate except for its rather conservative content of protein. We grow chicks on it without any evidence of inadequacy. The precipitating factor is parasitemia with a rapid surge from the moment of inoculation. That it is not the absolute intensity of the parasitemia but the attainment of a certain parasitemia in a certain period of time was brought out a number of times during the experiment; e.g., in group 1 of series 6, composed of 10 chicks on the G-ration which did not develop eyelid lesions, there were 4 individuals which attained on the 5th or 6th day parasitemias represented, respectively, by 49, 48, 61, and 82% of parasitized red blood cells, all in themselves of a magnitude sufficient to produce lesions, but they developed too slowly.

That the nature of the ration is as important as the parasite surge was also brought out clearly. The 4th-day parasite counts for group 1 of series 1, group 3 of series 3, group 4 of series 5, and groups 3 and 4 of 7 were as high or higher than those for the groups in the same series on the G-ration which became afflicted; but the eyes remained normal so far as could be determined macroscopically. Thus the nature of the ration is an important factor.

For some reason the lesions did not develop in any of the birds on S-ration, which was fairly comparable to G-ration in respect to biotin, pantothenic acid, and total protein content. The former contained 9 parts of soybean oil meal and 6 parts of linseed oil meal by weight in place of certain of the cereal products.

TABLE 1.—*The appearance of eyelid lesions in chicks on different diets.*

Series	Group	Regimen	No. in group	No. with eyelid lesions	Mean percent—4th day	Mean percent—5th day	Percent injected
1	1	S-R	3	0	55	51	2×10^8
	2	G-R	13	10	52	37	2×10^8
2	1	G-R	8	4 (slight)	48	60	1.5×10^8
	2	S-R	5	0	38	49	1.5×10^8
3	1	S-R	5	0	24	43	1×10^8
	2	G-R	5	5	30	52	1×10^8
	3	G-R+6 mg pant.	5	0	30	53	1×10^8
4	1	S-R	5	0	18	26	1×10^8
	2	G-R	5	0	10	11	1×10^8
5	1	G-R+2 μg bio.	5	0	44	49	1×10^8
	2	G-R	5	5	58	42	1×10^8
	3	S-R	5	0	28	44	1×10^8
	4	GS-R	5	0	59	58	1×10^8
	5	G-R+15 mg pant.	5	0	50	53	1×10^8
6	1	G-R	10	0	17	32	1×10^8
	2	G-R+1 μg bio.	10	0	19	33	1×10^8
	3	G-R+5 mg pant.	10	0	27	44	1×10^8
7	1	G-R	8	4	70	51	2×10^8
	2	G-R+0.5 μg bio.	5	0	66	47	2×10^8
	3	G-R+0.25 μg bio.	5	0	71	53	2×10^8
	4	G-R+0.5 mg pant.	5	0	73	50	2×10^8
	5	G-R	10	0	0	0	0
8	1	G-R	10	0	51	46	2×10^8
	2	G-R+0.5 mg pant.	5	0	67	63	2×10^8

Data pertaining to the conditions shown to affect the appearance of the eyelid lesions: G-R = G-ration; S-R = S-ration; GS-R = mixture of equal parts. G-R and S-R; Bio. = biotin administered once daily; Pant. = calcium pantothenate in 100 g of ration.

The answer to why the lesions did not appear in chicks on the S-ration is probably to be found in the composition of the two oil meals exclusive of biotin and pantothenic acid. Whatever the nature of the factor in S-ration that inhibits the development of the lesions, it is strong enough to function in a mixture of equal parts of the two rations.

It is of further interest that the incorporation of small amounts of biotin and pantothenate in the regimen of chicks on G-ration also prevented the disorder. This interchangeability of the two vitamins in playing a single objective role recalls the work of Emerson and Wurtz,[12] who reported that in rats fed a purified diet containing 0.5% succinylsulfathiazole the feeding of biotin mitigated the syndromes associated with lack of both biotin and pantothenic acid and, conversely, the feeding of pantothenic acid mitigated the syndromes associated with lack of both pantothenic acid and biotin (see also Everson et al[13]).

The paramount problem is, of course, how the malarial infection produces the disorder in chicks consuming G-ration. Trager[7] suggested that blood biotin is utilized to a certain extent by *P. lophurae*, and he showed that pantothenate prolonged the life of the same microorganism in vitro.[6] On the surface at least it would appear that the eyelid condition of the host is precipitated by the urgent biotin and/or pantothenic acid demand of a rapidly multiplying parasite population in the blood. It is evident that the lesions are the result of deficiency; but there are also considerations of special physiological activity of the host that must be faced—namely, regeneration of red cells in the erythropoietic tissues and the manufacture of antibody (opsonin) to the parasite.

We believe, however, that both alternative explanations can be ruled out, at least temporarily, for the following reasons: (1) The prodromal symptoms in group 2 of series 5 appeared on the 4th day, at which time the juvenile stages of erythrocytes, viz., basophil erythroblasts and polychromatophil erythroblasts, averaged only 0.6% of the total counts of red blood cells, indicating that the production of replacements for infected red blood cells had not yet proceeded very far; (2) a number of the chicks with incipient lesions on the 4th day showed an increase of parasitemia on the 5th and/or 6th day, indicating that the antibody titer of the blood was still negligible; (3) there exists no proof that biotin and/or pantothenic acid are utilized by the host in the manufacture of antibody (opsonin). It is to be pointed out here, however, that Trager[7] did find higher plasma and red cell levels of biotin on days 1½, 3, and 5 of *P. lophurae* infection in two normal 4-week-old chicks on an adequate diet, and suggested the possibility that it might play a role in reducing the numbers of parasites because it is mobilized in the blood stream during the infection in both chickens and ducks.

The sparing effect of the S-ration, comparable to the G-ration in its vitamin content, suggests that there may exist other factors than biotin and pantothenic acid which affect the development of the eyelid lesion. The effect of administrations of the individual essential amino acids, vitamin A, vitamin E, thiamin, riboflavin, niacin, choline, inositol, pyridoxine, folic acid, the newly discovered vitamin B_{12}—all these wait to be determined. The role, if any, of sunlight and of light waves

12. Emerson, G. A. and Wurtz, E. 1944, Proc. Soc. Exper. Biol. & Med. **57**: 47–49.
13. Everson G., Williams, E., Wheeler, E., Swanson, P., Spivey, M. and Eppright, M. 1948. J. Nutrition, **36**: 463–478.

of different length and intensity has not yet been investigated.

SUMMARY

An eyelid affliction is described, one characterized in its severest form by a process involving the encrustation and eroding away of the lower eyelid, and a healing process involving the regeneration of a replacement for the lost eyelid. The overgrowth may entirely, almost, or partially cover the eye. The milder form of the disorder produced a prominent notch in the margin of the lower lid. The development of lesion occurred under the following conditions: (1) The chicks were eating a ration ("G-ration") composed of 82% cereal grain products; (2) they were young, i.e., 12 or 14 days of age; (3) following inoculation they developed infections of *Plasmodium lophurae* of a certain intensity. It was not the absolute intensity of the parasitemia that precipitated the lesion but the attainment of a certain density within a certain period of time after inoculation. The evidence indicates that the disorder was the result of an acute dietary deficiency precipitated by competition for biotin and/or pantothenic acid between the host and a vigorous parasite population reproducing at its maximum rate. Supplementing the ration with small amounts of either of these vitamins forestalled the affliction. The suddenness of the onset and severity of the lesion, and the failure of a modification of the G-ration with almost identical biotin and pantothenic acid content (S-ration) to produce the lesion in infected birds, suggest that other factors also are concerned.

After this paper went to press there came to our attention a report by Couch et al[14] containing the statement that notched upper eyelids occurred in embryos and chicks from eggs injected with 5 units of insulin at 120 hours of incubation. The treatment presumably produced biotin deficiency, which in turn was reflected in a number of deformities, among them the notched eyelids.

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14. Couch, J. R., Cravens, W. W., Elvehjem, C. A. and Halpin, J. G. 1949, Poultry Sci. **28**: 276–282.

CHARACTERIZATION OF NEISSERIA ISOLATED FROM THE PHARYNGEAL REGION OF GUINEA PIGS

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For purposes of supplementing a collection of neisseria currently under investigation from the standpoint of systematics, an attempt was made to isolate additional strains from the bacterial flora of the pharyngeal region of normal guinea pigs. The present study describes a group of gram-negative diplococci which have been isolated from the pharyngeal area of guinea pigs. It is of interest to note that none of the currently recognized species of neisseria have been reported to be isolated from animals.[1] In addition, a review of the literature failed to reveal publications relative to neisseria from guinea pigs or other animals.

METHODS

Isolation of gram-negative diplococci.—Sterile cotton swabs, moistened with sterile physiological saline, were used to swab the pharyngeal region of apparently healthy guinea pigs. This swabbing technic was facilitated by the use of the instrument shown in figure 1, which, when inserted behind the upper and lower teeth of the animal, kept his mouth open. The swab was inserted through the opening into the pharyngeal area. The swabs were immediately streaked on trypticase soy agar plates. The plates were incubated for 48 hours at 35 C after which time they were examined and gram stains prepared from colonies suspected of being neisseria. Colonies found to consist of gram-negative diplococci were picked, restreaked on medium of the same composition, and after incubation, were checked for purity, gram reaction, and morphology. Each culture was restreaked several times to assure a pure culture of a gram-negative diplococcus.

Characterization of cultures isolated. Morphology.—On numerous occasions gram-stained preparations of all of the cultures selected by the technic described above were examined microscopically. All cultures were examined by phase contrast technic, and electron microscopic studies were made of a few. The latter examinations served to rule out short, gram-negative, bipolar rods which, in gram-stained preparations, are difficult to distinguish from diplococci.[2,3] The observations above were made from 18 to 24 hour cultures on trypticase soy agar slants incubated at 35 C.

FIG. 1.—Instrument employed to insert behind upper and lower teeth of guinea pig to facilitate swabbing of pharyngeal area.

Colonial characteristics.—Observations for colonial appearance were made from cultures streaked on trypticase soy agar plates and incubated 48 hours at 35 C.

Pigment.—Pigment production was observed from surface growth on Loeffler's slants after incubation for one week at 35 C. Additional observations were made by removing growth from a trypticase soy agar slant with a transfer loop and spreading it on a piece of white paper.

Hemolytic activity.—Hemolytic activity was

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1. Breed, R. S., Murray, E. G. D. and Hitchens, A. P. 1948, Bergey's Manual of Determinative Bacteriology, ed. 6, Baltimore, Williams and Wilkins Co.
2. Henriksen, S. D. 1947, Acta path. **24**: 184–197.
3. Schaub, I. G. and Hauber, F. D. 1948, J. Bact. **56**: 379–385.

determined by streaking the cultures on 5% rabbit blood agar plates and incubating for 48 hours at 35 C. Additional observations on hemolysin production were made using the tube hemolysin technic for demonstration of filterable hemolysin.

Oxidase test.—The oxidase test was performed on colonies from both blood agar and trypticase soy agar plates after 48 hours' incubation using the para-aminodimethylaniline monohydrochloride reagent.

Biochemical activity.—The cultures isolated were tested for their ability to ferment 5 carbohydrates, namely, glucose, fructose, maltose, sucrose, and mannite. None of the carbohydrates were autoclaved. They were made up individually in concentrated solution (10%) and sterilized by filtration through sintered glass filters. The base medium, cystine trypticase agar,[4] which is semi-solid, was sterilized at 10 to 12 pounds pressure (115 to 117 C) for 15 minutes, after which the respective sterile carbohydrate solutions were added aseptically to give a final concentration of 0.5%. Following inoculation, the tubes were incubated at 35 C and the reactions recorded after 24 and 48 hours and again at the end of 7 days' incubation.

RESULTS

Twenty-one cultures of gram-negative diplococci were isolated from the pharyngeal region of 43 guinea pigs examined. The morphological, cultural, and biochemical characteristics of this group of organisms may be described as follows:

Morphology.—All cultures were found to stain uniformly gram-negative. Gram-stained films showed the organisms to be arranged predominantly in pairs with adjacent sides flattened, though small clusters of spherical cells were frequently present. Examination of cultures by the phase contrast technic proved to be a very useful adjunct. The cellular morphology could be ascertained much more accurately by this method than from the gram-stained films. In preliminary observations, several cultures suspected of being gram-negative diplococci were found to be coccobacillary when observed by phase contrast. These cultures were discarded. As has already been indicated, very short gram-negative rods, which exhibit bipolar staining, have been mistaken for neisseria.

Some of the cultures were examined with the electron microscope. The morphological similarity between the strains isolated from guinea pigs and recognized species of *Neisseria* from a stock collection can be seen in figure 2.

Colonial characteristics.—The colonial appearance of all the cultures isolated was fairly uniform. Colonies averaged about 2 mm in diameter; they were circular with entire edges and convex with smooth glistening surfaces. Consistency of the colonies was usually butyrous, sometimes becoming viscid. Colonial development on trypticase soy agar was rapid, good growth occurring within 18 to 24 hours.

Pigment.—Each of the 21 cultures was characterized by a pigment ranging from a "light-caramel" to a "dirty-brown" color. This pigmentation was very distinct from the grayish-white appearance characteristic of *Neisseria catarrhalis* and *Neisseria sicca* and the yellow to yellowish green pigment characteristic of the chromogenic group.

Biochemical activity.—All 21 of the cultures were uniformly negative with regard to fermentative ability. None of the 5 carbohydrates tested were fermented by any of the cultures.

All of the cultures gave an immediate and distinct positive oxidase test with the para-aminodimethylaniline monohydrochloride reagent.

Hemolysis.—Four of the 21 cultures were weakly hemolytic against rabbit blood by the plate technic, and 3 of these produced a filterable hemolysin. The remaining cultures were nonhemolytic and did not effect any greenish discoloration of the blood.

4. Vera, H. D. 1948, J. Bact. **55**: 531–536.

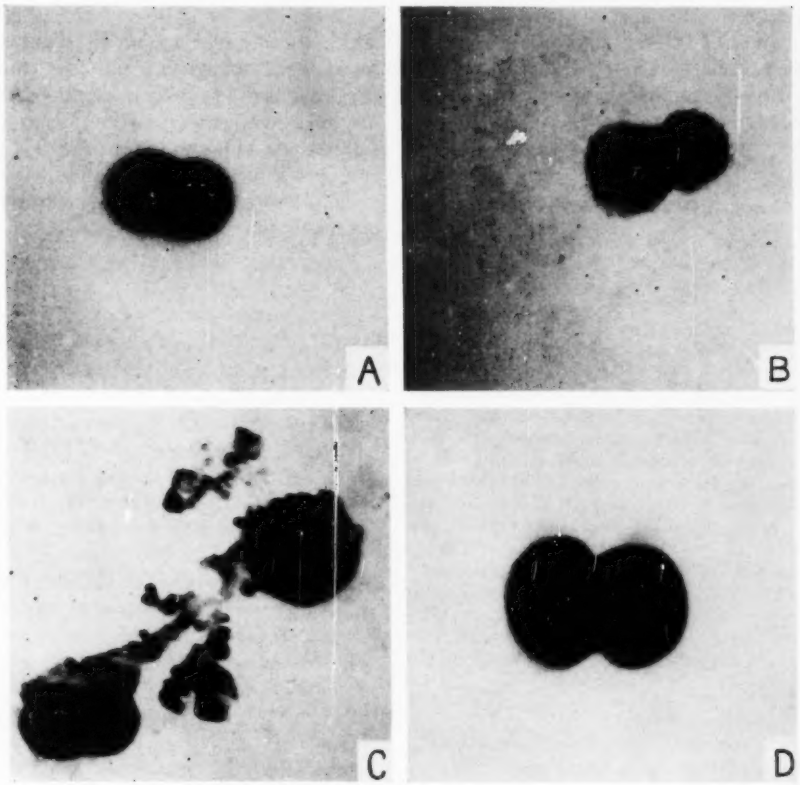

FIG. 2.—Electron micrographs of 4 cultures of neisseria. A. *Neisseria sicca*, ×28,500. B. *Neisseria catarrhalis*, ×28,500. C. Culture GP-11 from guinea pig, ×28,500. D. Culture R from guinea pig, ×40,800.

SUMMARY

The 21 cultures of gram-negative diplococci isolated from the pharyngeal area of guinea pigs constitute a rather homogeneous group from the standpoint of morphological, cultural, and biochemical characteristics. Since not all of the colonies were picked from any one plate, it is indeed possible that other gram-negative diplococci might have been present, though much less predominant than the type isolated.

Allocation of the gram-negative diplococci described in this report to a recognized species of the genus *Neisseria* presents difficulties. Their characteristics do not conform exactly with any of the recognized species of *Neisseria*. The biochemical reactions of this group conform to those described for *N. catarrhalis* and *Neisseria flavescens*.[1] However, the distinctness and uniformity of pigmentation of the guinea pig cultures do not conform with either of these two species. A few of the guinea pig cultures have been checked serologically against *N. flavescens* antiserum and no relationship was observed. Experiments are now in progress with a view toward elaborating upon the antigenic relationship of this

group to other species of *Neisseria*.

In view of the prevailing disagreement relative to the classification of chromogenic neisseria,[5] no attempt has been made to give species designation to this group of gram-negative diplococci. Additional data on the chromogenic species of *Neisseria* as a whole is needed in order that some agreement be arrived at with respect to the taxonomic significance which should be assigned to such properties as pigment production, colonial morphology, and fermentation of carbohydrates.

5. Wilson, G. S. and Miles, A. A. 1946, Topley and Wilson's Principles of Bacteriology and Immunity, Baltimore, Williams and Wilkins Co.

INCLUSION BODIES FROM DISEASES OF UNDETERMINED ETIOLOGY AND FROM APPARENTLY HEALTHY ANIMALS AS SEEN UNSTAINED WITH THE PHASE MICROSCOPE

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Small differences in refraction or thickness between structures can be used in phase microscopy to demonstrate without staining intracellular constituents that are invisible or indistinct in the ordinary, brightfield microscopy and even in darkfield or ultraviolet microscopy Thus, chromosomes (Michel, 1941; Bosshard, 1944), Golgi apparatus (Brice et al 1946; Jones, 1947a), mitochondria (Albertini, 1946; Jones, 1947b; and Zollinger, 1948), etc. have been studied in living or unstained, fixed cells. Zollinger (1948) observed inclusions in unstained tumor cells. Angulo et al (1949) and Angulo (1949) have reported the appearance of viral inclusion bodies in unstained tissue sections. In this paper, the appearance of inclusion bodies from diseases of undetermined etiology and from apparently healthy animals is reported.

MATERIALS AND METHODS

The material studied consisted of (a) oral mucosa from a cow with clinical and pathological pictures of malignant catarrhal fever (bovine epitheliosis); (b) lung from an adult human with inclusion-disease pneumonitis; (c) lung from an infant with Hecht's giant cell pneumonia; (d) lung from another infant with Hecht's giant cell pneumonia; (e) lung from another infant with Hecht's giant cell pneumonia; (f) lung from a dog with giant cell pneumonia; (g) spleen from a turkey poult dying from an unknown cause. Inclusion bodies were noticed in this specimen by Jungherr (1948), although they have not yet been reported; (h) throat epithelium from an apparently healthy human; (i) pancreas from an apparently healthy, laboratory rat; (j) pancreas from an apparently healthy chicken; (k) pancreas from an apparently healthy turkey. Lucas (1948) observed inclusions in this specimen which have not yet been reported.

The specimens examined had been studied before by other workers who used standard techniques. Reports of these studies have appeared in the literature, except for the case of specimens g and k. Those reports will be cited here in describing the appearance of the corresponding inclusion body under the phase microscope.

The techniques employed have been described before (Angulo et al, 1949; Angulo, 1949). One smear of throat epithelium was fixed with methanol and mounted unstained in paraffin oil. Another smear was stained with Giemsa.

OBSERVATIONS

Inclusion bodies of malignant catarrhal fever (Goss et al, 1947) were readily seen. They appeared as finely granular masses of rather irregular shape (fig. 1). Sometimes they showed vacuoles. Structural details showed nicely. Contrast was sharp due to the dark yellow color of the inclusion bodies when examined with the bright contrast of the phase microscope and also to the presence of a narrow, transparent zone around the inclusion bodies. The intracytoplasmic position of the latter was clearly seen. Inclusion bodies could be differentiated from the shriveled nuclei of some cells on account of their granular structure, the narrowness of the clear zone surrounding them and by the absence of folds.

In the specimen of inclusion-disease pneumonitis (McMillan, 1947), the

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intranuclear and intracytoplasmic inclusion bodies were sufficiently conspicuous so that the cells which contained them were readily recognizable (fig. 2, 3 and 4). Bright contrast of the phase microscope produced a yellow color in the intranuclear inclusion bodies and revealed their granular character. The background of nucleoplasm was transparent and structureless and extended beyond the inclusion to give the appearance of a typical halo. Intracytoplasmic inclusion bodies were seen as granules of large size and rather angular outlines. They were conspicuous against the cytoplasmic background. Dark-contrast phase microscopy was inferior to bright-contrast phase microscopy in the examination of the specimen.

The identification and study of the inclusion bodies from the specimens of Hecht's giant cell pneumonia (Pinkerton et al, 1945) was an easy task. In the first specimen studied using bright-contrast phase microscopy, intranuclear inclusion bodies were conspicuous because they occupied a space that looked transparent in the nuclei that did not contain inclusion bodies. In some cases, these inclusion bodies showed structural details such as small granules as well as larger granules surrounded by a clear zone. They were similar to some of the inclusion bodies found in the specimen of inclusion-disease pneumonitis, in an adult. Intranuclear inclusion bodies in dark-contrast phase microscopy were somewhat more conspicuous than shown in bright-contrast phase microscopy. The reverse was true with intracytoplasmic inclusion bodies. The latter (fig. 7, 10 and 11) were recognized easily due to their contrast and morphological characteristics. Structural details were seen better in this unstained section in dark-contrast phase microscopy than in the control stained preparation examined with the ordinary, brightfield microscope.

In the second specimen of Hecht's giant cell pneumonia, granules were observed in an intracytoplasmic inclusion body. Those granules stood out against the ochre yellow background. Elongated granules were seen in intranuclear inclusion bodies (fig. 12). These granules showed a sharp contrast with the remainder of the inclusion body. Bright-contrast phase microscopy was more efficient than dark-contrast phase microscopy for studying intranuclear inclusion bodies, which was the reverse of that found in the preceding specimen. In the third specimen of Hecht's giant cell pneumonia the findings were similar either to those made in the first or in the second previous specimen.

The inclusion bodies of dog giant cell pneumonia (Pinkerton et al, 1945)

PLATE 1

In most of the figures, only the inclusion bodies are in focus.

FIG. 1.—Oral mucosa from a cow with malignant catarrhal fever. Notice intracytoplasmic inclusion body, ib. Unstained, bright-contrast phase microscope. ×920.

FIG. 2.—Lung from an adult human with inclusion-disease pneumonitis. An intranuclear inclusion body, ib. Unstained, bright-contrast phase microscope. ×920.

FIG. 3.—Same specimen of figure 2. Large intranuclear inclusion body, ib. Unstained, bright-contrast phase microscope. ×920.

FIG. 4.—Same inclusion body shown in figure 2 but photographed with a dark-contrast phase microscope. ×920.

FIG. 5.—Same specimen of figures 2, 3 and 4 but stained with hematoxylin and eosin and photographed with an ordinary, brightfield microscope. Large intranuclear inclusion body, ib. ×920.

FIG. 6.—Same inclusion body shown in figures 2 and 4 but photographed with an ordinary, brightfield microscope. ×920.

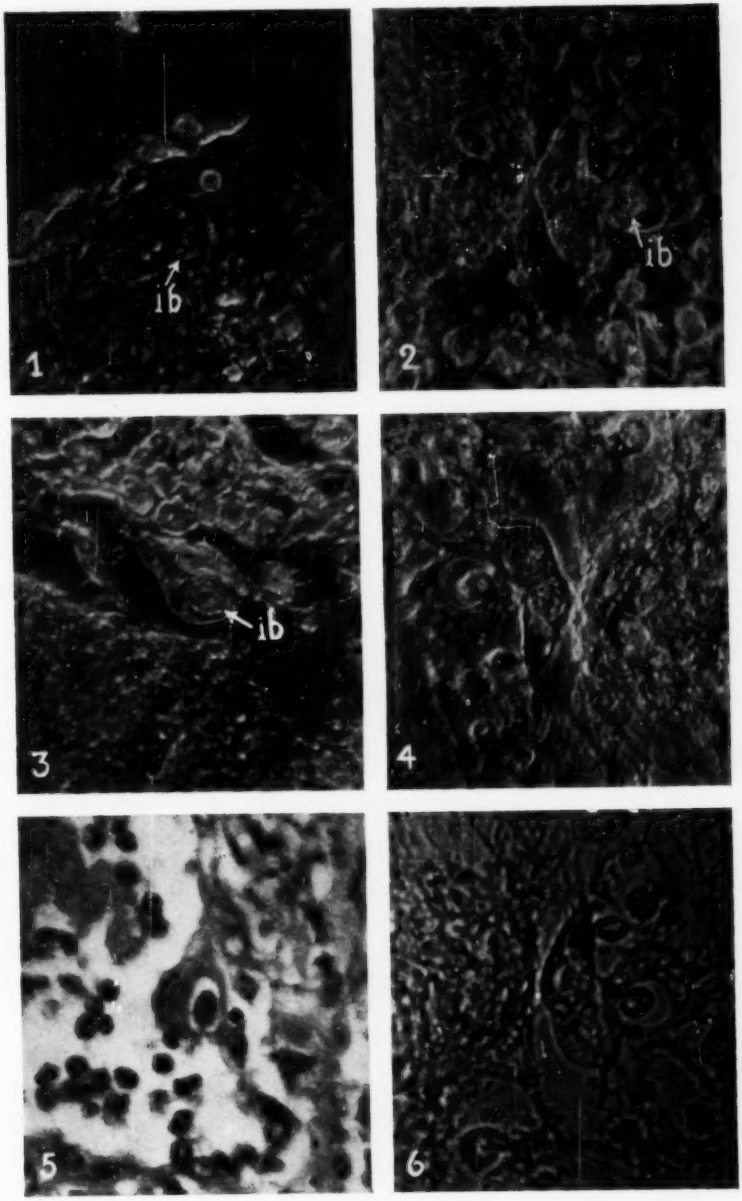

PLATE 1

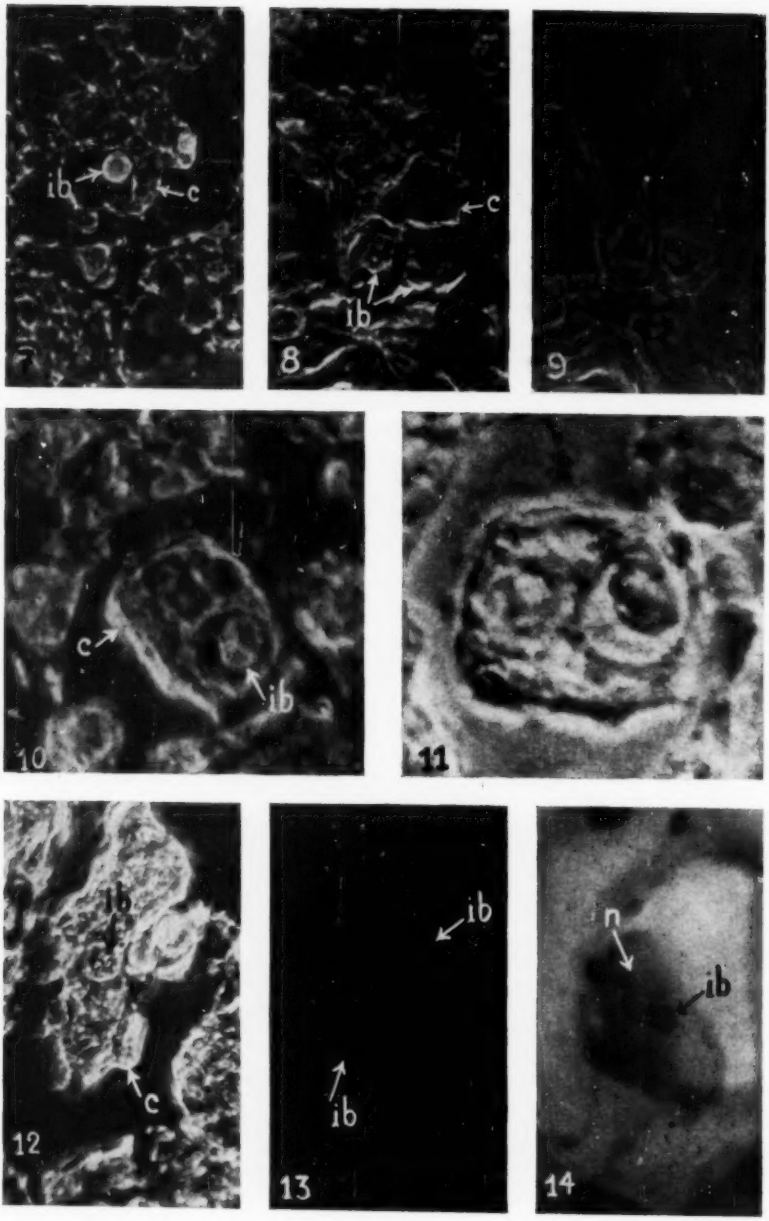

PLATE 2

looked similar (fig. 8 and 9) to those of the specimen from human cases. Besides vacuoles as well as granules were observed in several intranuclear inclusion bodies. In one of these intranuclear inclusion bodies, large vacuoles harbored ill-defined rods of opaque material which showed lower refractivity than the rest of the inclusion body. Intracytoplasmic inclusion bodies were small, slightly elongated and looked somewhat different than common granules. The former were greenish in bright-contrast phase microscopy and stood out in sharp contrast against the cytoplasm. They were surrounded by a clear, transparent zone.

The inclusion bodies of the specimen of turkey poult spleen (Jungherr, 1948) were easily recognized (fig. 19 and 20). Their presence in nuclei was conspicuous. Some inclusion bodies were homogeneous while others were granular. Inclusion body outlines were distinct, and structural details were clearly seen. Besides all these characteristics, the marked refractivity of the inclusion bodies helped in their identification.

The inclusion bodies in cells of throat epithelium (Broadhurst et al, 1936) from an apparently healthy human were one of the easiest to detect in the material examined. They were usually oval (fig. 13), and some of them showed a dark, round particle at the center. A few others were finely vacuolated. A halo, apparently due to a diffraction phenomenon, was prominent around some inclusion bodies. This halo helped in making the inclusion bodies more conspicuous, especially in bright-contrast phase microscopy. A more refractive area could be clearly seen in some of those bodies. In dark-contrast phase microscopy, inclusion bodies were lighter in color than the cytoplasm and more refractive. Other inclusion bodies were large and oval (fig. 13). These showed a well defined internal structure in which darker zones, apparently vacuoles, were observed. Around one part of these, a seemingly empty zone was noticed. No structure could be detected in small inclusion bodies.

Intracytoplasmic inclusion bodies in the pancreas from an apparently healthy laboratory rat (Duff and Cor

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PLATE 2

In most of the figures, only the inclusion bodies are in focus.

FIG. 7.—Lung from an infant with Hecht's giant cell pneumonia. A giant cell, c, with an intracytoplasmic inclusion body, ib. Unstained, bright-contrast phase microscope. ×920.

FIG. 8.—Lung from a dog with giant cell pneumonia. A giant cell, c, with two intranuclear inclusion bodies, ib. Unstained, bright-contrast phase microscope. ×920.

FIG. 9.—Same field of figure 8 but photographed with a dark-contrast phase microscope. ×920.

FIG. 10.—Lung from an infant with Hecht's giant cell pneumonia. A giant cell, c, with an intracytoplasmic inclusion body, ib, surrounded by a large vacuole. Nuclei of the giant cell not in focus. Unstained, bright-contrast phase microscope. ×1500.

FIG. 11.—Same giant cell of figure 10 but photographed with a dark-contrast, phase microscope under a higher magnification. ×2860.

FIG. 12.—Lung from an infant with Hecht's giant cell pneumonia. A granular, intranuc'ear inclusion body, ib, is seen in a giant cell, c. Other nuclei out of focus. Unstained, bright-contrast phase microscope. ×920.

FIG. 13.—Smear of throat exudate from an apparently healthy human. Two intracytoplasmic inclusion bodies, ib, are seen in an epithelial cell. Notice structural details in the larger inclusion body. Unstained, dark-contrast phase microscope. ×920.

FIG. 14.—Same specimen of figure 13 but stained with Giemsa and photographed with an ordinary, brightfield microscope. The nucleus, n, is barely evident. Intracytoplasmic inclusion body, ib. ×920.

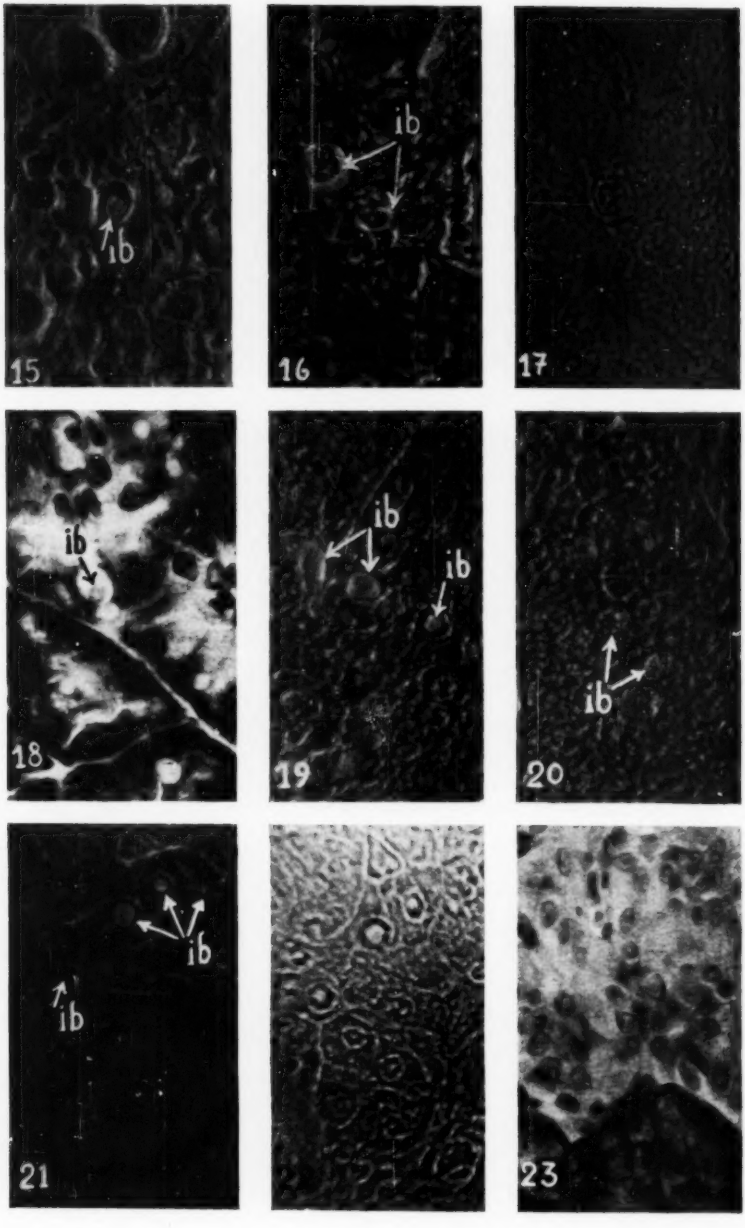

PLATE 3

bett, 1945) were not easy to identify in bright-contrast phase microscopy because they did not show much contrast in most cases. Dark-contrast phase microscopy was better for the identification and study of these inclusion bodies (fig. 16). With the latter contrast, inclusion bodies appeared darker than the cytoplasm and stood out in good contrast. Granules inside inclusion bodies were dark in bright-contrast phase microscopy, which was an unusual finding. The clear zone around the inclusion bodies, the internal structure of the latter, their shape and their size helped in identification.

The intranuclear inclusion bodies in the pancreas from an apparently healthy chicken (Lucas, 1947) were readily seen. They showed the sharpest contrast of all inclusion bodies studied. They were seen as almost homogeneous, opaque, whitish, round masses of distinct contours (fig. 21). Careful focusing revealed small particles of varied refractivity in the inclusion bodies. The latter occupied a great part of the nuclei that harbored them. On account of this and the opacity of the inclusion bodies, those nuclei sharply differed in appearance from the normal nuclei. Bright-contrast phase microscopy revealed the inclusion bodies better than dark-contrast phase microscopy.

The intranuclear inclusion bodies in the pancreas from an apparently healthy turkey (Lucas, 1948) varied in structure. Some were slightly refractive, coarsely granular masses of irregular contours. Other inclusion bodies were finely granular, mat, and showed a small refractive granule at the center. Identification of inclusion bodies was easy in this specimen. Dark-contrast phase microscopy was inferior to bright-contrast phase microscopy.

DISCUSSION

The studies reported here and elsewhere (Angulo et al, 1949; Angulo, 1949) have been made on unstained specimens from 14 diseases of established viral etiology, from 5 diseases of viral etiology not established and from 4 animals showing no sign of disease. Two plant tissue specimens were included in the material studied. Identification and structural study of all inclusion bodies were easily done. Granules, vacuoles and other structural details of these inclu-

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PLATE 3

In most of the figures, only the inclusion bodies are in focus.

FIG. 15.—Pancreas from an apparently healthy laboratory rat. An intracytoplasmic inclusion body, ib, is surrounded by a vacuole. Unstained, bright-contrast phase microscope. ×920.

FIG. 16.—Another field from the same specimen of figure 15. Notice two intracytoplasmic inclusion bodies, ib. A vacuole surrounds each inclusion body. Unstained, dark-contrast phase microscope. ×920.

FIG. 17.—Same field of figure 16 but photographed with an ordinary, brightfield microscope. ×920.

FIG. 18.—Same specimen of figures 15 and 16 but stained with hematoxylin and eosin and photographed with an ordinary, brightfield microscope. ×920.

FIG. 19.—Spleen from a turkey poult dying from unknown cause. Notice homogeneous, intranuclear inclusion bodies, ib. Unstained, bright-contrast phase microscope. ×920.

FIG. 20.—Another field from the same specimen of figure 19. Notice granular, intranuclear inclusion bodies, ib. Unstained, bright-contrast phase microscope. ×920.

FIG. 21.—Pancreas from an apparently healthy chicken. Notice homogeneous as well as slightly granular, intranuclear inclusion bodies, ib. Unstained, bright-contrast phase microscope. ×920.

FIG. 22.—Same field of figure 21 but photographed with an ordinary, brightfield microscope. ×920.

FIG. 23.—Specimen of figures 21 and 22 but stained with hematoxylin and eosin and photographed with an ordinary, brightfield microscope. ×920.

sion bodies appeared neatly in the phase microscope, provided that tissue sections or smears were not too thick. Intracellular position, shape and even some color could also be easily recognized.

In the study of structural details, phase microscopy of unstained inclusion bodies was quite comparable to ordinary, brightfield microscopy of hematoxylin-eosin-stained inclusion bodies. In some cases, the unstained inclusion bodies showed sharper structural details or more contrast than the stained, control inclusion bodies. When, in the unstained preparations studied, a change from phase to brightfield microscopy was made, the identification of inclusion bodies became either difficult or impossible.

The medium-bright contrast diffraction plate was found superior to the medium-dark contrast plate for the identification and structural study of the majority of the inclusion bodies examined. It was better also for histological study of most of the tissues harboring the cells showing the inclusion bodies.

SUMMARY

Bright-contrast and dark-contrast phase microscope studies of the inclusion bodies found in unstained specimens from diseases of undetermined etiology and from apparently healthy animals are reported. The specimens studied were obtained from malignant catarrhal fever of cattle, inclusion disease, Hecht's giant cell penumonia, dog giant cell pneumonia, spleen from a turkey poult dying from unknown cause, throat epithelium from an apparently healthy human, pancreas from an apparently healthy laboratory rat, pancreas from an apparently healthy chicken and pancreas from an apparently healthy turkey.

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ACTIVE IMMUNIZATION OF MICE WITH ULTRAVIOLET-INACTIVATED LYMPHOCYTIC CHORIOMENINGITIS VIRUS VACCINE AND RESULTS OF IMMUNE SERUM THERAPY

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Attempts to immunize mice against lymphocytic choriomeningitis virus have been successful only when active virus was employed.[1-3] Traub[2] has reported partial immunity in guinea pigs vaccinated with formalinized vaccines prepared from infected guinea pig tissues. In unpublished experiments[4] we were unable to immunize mice or monkeys with infected guinea pig brain or spleen suspensions inactivated by minimal amounts of heat or formalin. We also found that rhesus monkeys and guinea pigs inoculated subcutaneously with subinfective amounts of the J. P. strain of choriomeningitis virus[5] remained susceptible to reinoculation with approximately 10 LD_{50} of homologous virus given by the same route at intervals of one to three weeks.

This paper deals with (1) the preparation of a completely inactivated lymphocytic choriomeningitis vaccine by means of ultraviolet irradiation and (2) the therapy of infected mice and guinea pigs with hyperimmune or convalescent choriomeningitis antiserum. The technic of irradiation used is described in previous publications[6,7] and consists essentially of exposing continuously flowing thin (less than 1 mm) films of virus suspensions to the Oppenheimer-Levinson type lamp, which is a powerful source of total and extreme ultraviolet energy. By means of this technic turbid suspensions of many kinds of bacteria and viruses can be completely inactivated by irradiation for less than one second with a minimal loss of antigenicity.[6-9] A preliminary report of the present paper has been published previously.[10]

MATERIALS AND METHODS

Virus.—The J. P. strain of lymphocytic choriomeningitis virus was used.[5] This strain was maintained by serial subcutaneous passages in guinea pigs, and its identity was verified periodically by neutralization tests with known choriomeningitis antiserum. The LD_{50} titer was 10^{-6} when inoculated intracerebrally into Swiss mice or subcutaneously into guinea pigs weighing 300 to 400 g. Rhesus monkeys (*Macaca mulatta*) uniformly succumbed to subcutaneous inoculation (0.5 cc) with 10^{-4} dilutions of this strain. Further details of the properties of this strain are described elsewhere.[5]

Preparation of irradiated virus vaccine.—Suspensions for irradiation were prepared from the cerebral tissues of guinea pigs dying from infection with the J. P. strain of virus and stored at −76 C. The infected brains were ground in a cooled Waring blendor and diluted with physiological salt solution to make a 4% suspension by weight. Before irradiation the suspension was filtered through 120-mesh pongee in order to

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remove those tissue particles which escaped being ground sufficiently fine.

Four percent uncentrifuged brain suspensions infected with the choriomeningitis virus were completely inactivated by irradiation for 0.3 seconds. Only bacteriologically sterile suspensions were used for vaccine preparation, and the irradiated vaccines were preserved with 1:10,000 merthiolate and stored at 5 C. Several separate lots of completely inactivated vaccines were prepared and tested for immunogenic potency in both Swiss mice and rhesus monkeys. Proof of inactivation consisted of inoculating Swiss mice intracerebrally (0.03 cc) and guinea pigs both intracerebrally (0.2 cc) and subcutaneously (2 cc) with undiluted irradiated vaccines and serial passage of the brains of randomly sacrificed animals on the 10th day after inoculation.

TABLE 1.—*Vaccination of mice with irradiated lymphocytic choriomeningitis vaccine.*

Experiment	Vaccine	Dilutions of challenge virus injected intracerebrally in 0.03 cc amounts*					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	Irradiated 0.3 second	8/8	7/8	3/8	2/8		
	Unvaccinated controls	8/8	8/8	8/8	8/8	6/8	0/8
2	Irradiated 0.3 second	5/5	4/6	2/7	3/7		
	Unvaccinated controls	8/8	7/7	7/7	7/7	5/7	0/7

* Numerator = number of mice which developed choriomeningitis and died. Denominator = number of mice inoculated.

EXPERIMENTAL RESULTS

Active immunization of mice and monkeys.—Swiss mice of a single strain, 4 to 5 weeks of age, were used. Groups of mice equally divided according to sex and weight were injected intraperitoneally with three 0.5 cc doses of undiluted irradiated vaccine (4%) given at weekly intervals. Twenty-one days after the first dose of vaccine, the mice were challenged intracerebrally with serial 10-fold dilutions of homologous virus diluted in nutrient broth (pH 7.4). Results obtained in the two experiments outlined in table 1 show that the vaccinated mice resisted at least 200 LD_{50} of virus.

Five rhesus monkeys weighing 3 to 4 kg were vaccinated in a similar manner and resisted approximately 10 LD_{50} of virus when challenged 21 days after the first dose of vaccine by the subcutaneous route.

Therapy of mice with hyperimmune serum.—In preliminary work we were unable to prepare a potent hyperimmune serum in rabbits repeatedly inoculated with irradiated or active virus given by the subcutaneous, intraperitoneal or intravenous routes. The most protective rabbit antiserum prepared when undiluted neutralized only 1000 LD_{50} of virus. The neutralizing titer was determined by injecting Swiss mice intracerebrally with mixtures of equal parts of undiluted serum and serial 10-fold virus dilutions in physiological salt solution incubated at 37 C for one hour prior to inoculation. Six mice were inoculated with each serum-virus mixture.

A potent hyperimmune monkey serum was prepared by repeated injections of irradiated vaccine followed by active choriomeningitis virus. A total of 12 weekly 1 cc injections were given according to the following schedule of immunization: Each monkey received three subcutaneous doses of undiluted irradiated vaccine. On the 21st day the monkeys were inoculated subcutaneously with approximately 10 LD_{50} of active virus. This amount was increased 10-fold each week until they resisted the inoculation of a 10% suspension of active virus (approximately 10,000 LD_{50}). The monkeys were then given 5 additional injections of 10% active suspension and were bled two weeks after the last injection. The titer of the pooled hyperimmune serum was such that a 100-fold dilution in physiological salt

solution readily neutralized an equal volume of 10% virus suspension.

Swiss mice weighing about 17g were used in serum therapy experiments. A total of 72 mice were injected intracerebrally with approximately 5 LD_{50} of virus. Twelve mice were given 1 cc of hyperimmune serum by the intraperitoneal route simultaneously while an equal number of control mice were injected with normal monkey serum. At intervals of 24 hours, other groups of 12 mice were inoculated with 1 cc of hyperimmune serum intraperitoneally up to 96 hours after virus inoculation. The first signs of choriomeningitis generally appeared on the 6th day following virus inoculation.

TABLE 2.—*Treatment of choriomeningitis-infected mice with hyperimmune monkey serum.*

Time between virus inoculation and serum therapy*	Number of animals	Number of mice surviving	Day of death of mice
Simultaneously	12	12	–, –, –, –, –, –, –, –, –, –, –, –
24 hr.	12	8	7, 7, 8, 8, –, –, –, –, –, –, –
48 hr.	12	5	6, 7, 7, 8, 8, 8, 9, –, –, –, –, –
72 hr.	12	0	6, 6, 7, 7, 7, 7, 7, 8, 8, 8, 9, 9
96 hr.	12	0	6, 6, 7, 7, 7, 7, 7, 7, 7, 8, 8, 8
Controls†	12	0	6, 6, 6, 6, 7, 7, 7, 7, 7, 8, 8, 8

* Inoculated with 1 cc of hyperimmune serum intraperitoneally.
† Inoculated with 1 cc of normal monkey serum intraperitoneally.

The results of this serotherapy experiment as summarized in table 2 show that there was definite protection when serum was given as long as 48 hours after virus injection. Forty-two per cent of the treated animals survived, whereas the mortality rate in the controls was 100%.

Therapy of guinea pigs with convalescent serum.—Although the following experiment on serotherapy of guinea pigs with human convalescent serum obtained from a laboratory infection of lymphocytic choriomeningitis[11] was done several years ago, we feel it is worth reporting because the results obtained parallel the present findings in mice. Human convalescent serum was collected about 5½ months after onset of illness, at which time the choriomeningitis-neutralizing antibody titer had reached its peak. The undiluted human serum neutralized approximately 10,000 MLD of virus. A total of 66 guinea pigs weighing 300 to 400 g were injected subcutaneously with approximately 5 MLD of virus. Six pigs were given 5 cc of convalescent serum simultaneously while 6 controls were injected with human serum known to contain no choriomeningitis antibodies. Thirty additional virus-inoculated guinea pigs receiving no serum and used in other experiments also served as controls. Separate groups of 6 animals each of the remainder were inoculated with 5 cc of convalescent serum intraperitoneally at intervals of 24, 48, 72 and 96 hours, respectively, after virus inoculation.

TABLE 3.—*Treatment of choriomeningitis-infected guinea pigs with human convalescent serum.*

Time between virus inoculation and serum therapy*	Number of animals	Incubation period	Average length of life in days	Number of animals surviving
Simultaneously	6	—	—	6
24 hr.	8	8.5	17	0
48 hr.	6	7.0	15.5	0
72 hr.	6	3.5	10	0
96 hr.	6	3.5	9	0
Controls†	36	3.5	9	0

* Inoculated with 5 cc of convalescent serum intraperitoneally.
† Includes 6 animals each of which received 5 cc of human serum known to contain no choriomeningitis antibodies intraperitoneally.

Results given in table 3 show that only those animals that were given

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serum simultaneously with virus inoculation survived. There was, however, a significant prolongation of incubation period and life span in guinea pigs treated with serum as long as 48 hours after infection. Animals treated 72 and 96 hours, respectively, after virus inoculation had the same incubation period and length of life as the controls.

DISCUSSION

The results of the present experiments in which mice were vaccinated with irradiated choriomeningitis virus indicate that significant intracerebral immunity was developed. Previous attempts to immunize mice with formalin, heat, oleic acid or ether-inactivated virus were uniformly unsuccessful.[1–4] The degree of active immunity obtained with the irradiated choriomeningitis vaccine in mice was not as marked as we have previously reported for ultraviolet-inactivated rabies, St. Louis encephalitis and poliomyelitis vaccines.[5,7] Perhaps a relatively greater degree of active immunity could be elicited by use of a more immunogenic lymphocytic choriomeningitis virus than the strain (J.P.) which was used in the present studies.[12] We have already mentioned that subinfective amounts of this strain failed to produce active immunity in guinea pigs or monkeys.[4]

Traub[2] has reported partial immunity in guinea pigs vaccinated with formalin-inactivated choriomeningitis vaccines. This worker reported that a slight degree of active immunity could be achieved only when the formalinized vaccines contained a variety of infected guinea pig tissues. Vaccines prepared from infected brain tissue had practically no immunizing power for guinea pigs although they contained at least as much virus as other guinea pig tissues prior to inactivation. In contrast to these results, we have prepared effective vaccines for mice by ultraviolet irradiation of infected guinea pig brain tissues. The immunizing value of the irradiated vaccine was not tested in guinea pigs, but vaccinated rhesus monkeys resisted approximately 10 LD_{50} of virus given subcutaneously.

Our clinical report[13] on the successful treatment of a case of recurrent lymphocytic choriomeningitis with pooled normal adult serum prompted us to carry out animal experiments with immune serum therapy. The results obtained on therapy of mice with hyperimmune monkey serum show definite protection as long as 48 hours after virus inoculation. Forty-two percent of animals treated up to 48 hours after infection survived, while the fatality rate in the controls was 100%. There was also a significant prolongation of incubation period and life span in guinea pigs treated with convalescent serum as long as 48 hours after infection. Perhaps some of these animals would have survived if repeated dosages of the convalescent serum were administered or a more potent serum, such as the hyperimmune monkey serum used in mouse therapy experiments, had been employed.

There is increasing evidence that laboratory animals infected with various viruses can be successfully treated with hyperimmune serum. Levinson and Shaughnessy[14] have recently reviewed the value of immune serum in the prophylaxis and treatment of virus diseases. Animals previously infected with Western equine encephalomyelitis,[15,16]

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influenza virus,[17] poliomyelitis,[18,19] rabies[20] and herpes[21] have been successfully treated with hyperimmune serum. In fact, Zichis and Shaughnessy[16,17] were able to save guinea pigs visibly ill with Western equine encephalomyelitis by therapy with large and repeated injections of a potent hyperimmune rabbit serum.

SUMMARY AND CONCLUSIONS

1. Mice immunized with ultraviolet-inactivated lymphocytic choriomeningitis vaccine developed significant intracerebral immunity. Rhesus monkeys immunized in a similar manner resisted approximately 10 LD_{50} of homologous virus given subcutaneously.

2. Attempts to immunize mice or monkeys with heat or formalin-inactivated lymphocytic choriomeningitis vaccines were unsuccessful.

3. Mice treated with hyperimmune monkey serum showed definite protection as long as 48 hours after choriomeningitis virus inoculation. Forty-two percent of the treated animals survived, while the fatality rate in the controls was 100%. There was also significant prolongation of incubation period and life span in guinea pigs treated with a less potent human convalescent serum administered 48 hours after infection.

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MORPHOLOGICAL OBSERVATIONS BY ELECTRON MICROSCOPY OF THE HAMSTER-ADAPTED AND THE MOUSE-ADAPTED NEWCASTLE VIRUS AFTER CULTURE IN CHICK EMBRYOS

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Micrographs of purified egg-adapted Newcastle virus suspended in 0.07 to 0.15 *M* sodium chloride by Bang[1,2] showed that tailed or sperm-like forms predominated. Cunha et al[3] reported similar morphological characteristics. Reagen et al[4] also found sperm-like forms in the hamster-adapted Newcastle virus, California strain 11914, when the virus particles were suspended in normal saline.

Hamster-adapted Newcastle virus of the 249th intracerebral passage was cultivated in 10- or 11-day embryonated chicken eggs through three serial subinoculations or until 100% of the inoculated embryos died within 72 hours. A pool of infected allantoic fluid was harvested. The virus was readily neutralized by hyperimmune Newcastle antiserum and was pathogenic for 70% of 6-week-old New Hampshire chickens injected subcutaneously with 0.2 cc. Hamster-adapted virus of the 249th passage was nonpathogenic for chickens of the same age when the same route of injection was employed. One hundred cc of the pooled allantoic fluid was centrifuged in a horizontal centrifuge for 15 minutes at 1000 r.p.m. and then for 2 hours at 50,000 r.p.m. in a Spinco Ultra Centrifuge. The sediment was resuspended with saline, placed on parolodion prepared screens, shadowed with chromium at arc tan 1/6, and examined under the RCA Emu electron microscope. Small tailed bodies and a few nontailed forms were observed as shown in figure 1.

The same procedure was used for mouse-adapted[5] Newcastle virus of the 18th passage. After having been cultivated in embryonated chicken eggs for 7 passages or until 100% of the inoculated embryonated eggs died within 72 hours, the allantoic fluid produced typical symptoms of Newcastle disease in 83% of the 6-week-old New Hampshire chickens inoculated subcutaneously with 0.2 cc. The mouse-adapted virus of the 18th passage given in the same dose subcutaneously did not produce symptoms of Newcastle disease in the young chickens. This allantoic fluid was neutralized by hyperimmune Newcastle antiserum. After centrifugation the saline-suspended virus appeared with long, slender tails as shown in figures 2 and 3.

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SUMMARY

Hamster-adapted and mouse-adapted

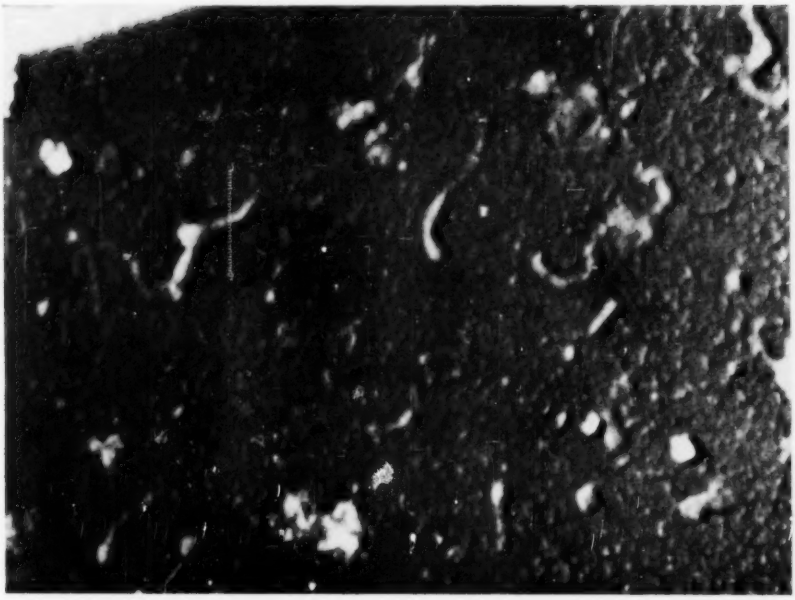

FIG. 1.—Hamster-adapted Newcastle virus in allantoic fluid of chick embryos, resuspended in saline, shadowed with 3 mg chromium at tangent 1/6. ×38,000.

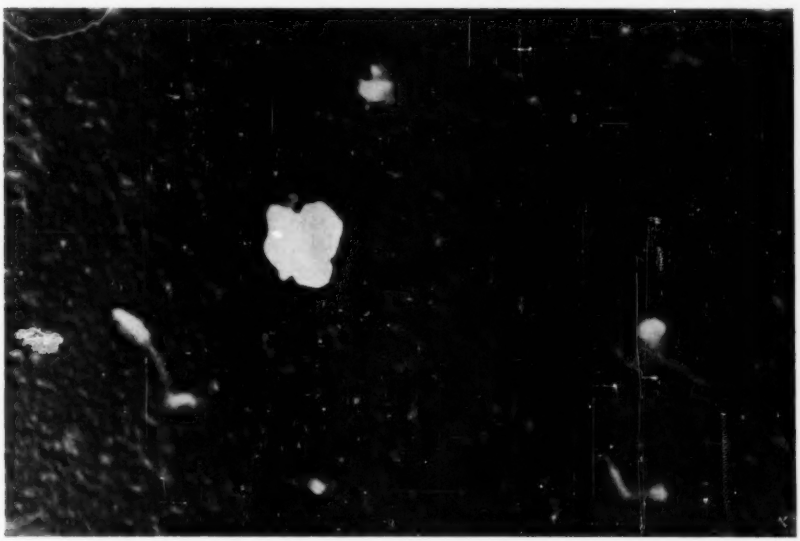

FIG. 2.—Mouse-adapted Newcastle virus in allantoic fluid of chick embryos, resuspended in saline, shadowed with 3 mg chromium at tangent 1/6. ×34,000.

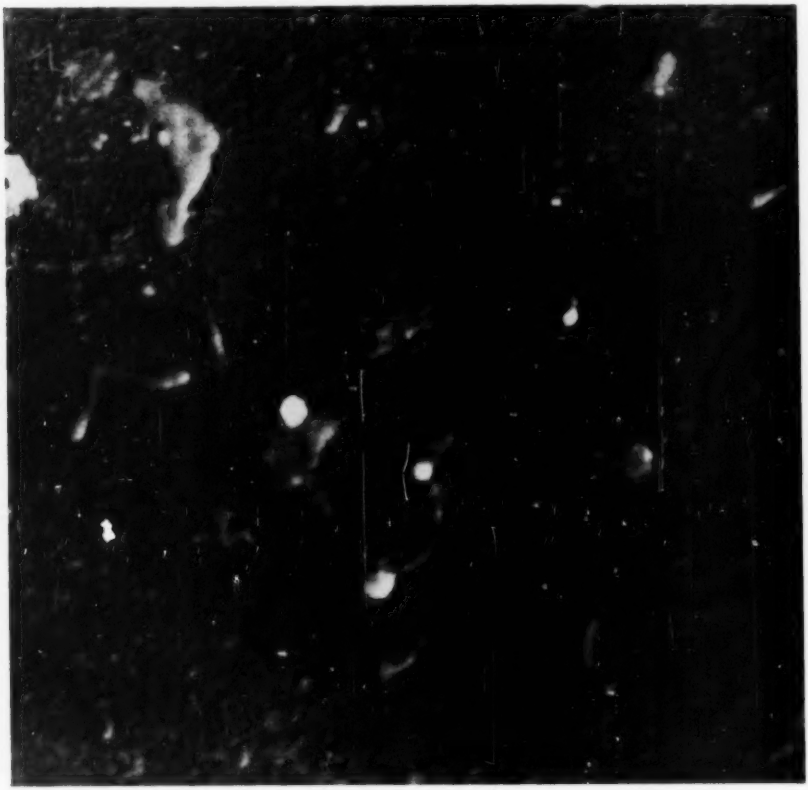

FIG. 3.—Mouse-adapted Newcastle virus in allantoic fluid of chick embryos, resuspended in saline, shadowed with 3 mg chromium at tangent 1/6. ×30,000.

Newcastle virus, California strain 11914, becomes pathogenic for young New Hampshire chickens after its growth is well established in embryonated chicken eggs. Electron micrographs of saline-suspended virus from the allantoic fluid infected with hamster-adapted or mouse-adapted virus appear similar to those particles observed in saline suspensions of the egg-adapted Newcastle virus. The predominating particles observed appear as sperm-like bodies with head piece and tails of varying lengths.

INDUCED LATENT INFECTION AND RESULTANT ACTIVE IMMUNITY TO M M MOUSE ENCEPHALOMYELITIS VIRUS IN MICE SUCKLED BY IMMUNE FOSTER MOTHERS

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Previously reported experiments[1] with M M strain of mouse encephalomyelitis virus showed that newborn mice were passively protected against that infection for about two months when suckled by the immune mother for 21 days. Unmodified virus administered during the first month was without demonstrable effect, either clinically or in prolonging immunity. Virus administered during the second month in amounts lethal for the progeny of normal mice gave rise to an active persistent immunity through inapparent infection. Proof of successful active immunization came first from continued ability to withstand ordinarily lethal amounts of virus, and secondly from the ability of female mice to transmit protection passively to the immediately subsequent generation. The mechanism of the passive immunity transmitted from mother to offspring was not determined. An incident in the course of these observations indicated that it was largely a function of antibodies transmitted by milk. The matter had to do with a normal mouse and her litter of 7 sucklings.

Three of the litter were injected with M M virus and all died—the regular and expected circumstance. The mother ate two of them, with no demonstrable clinical effect. This mouse continued to nurse her 4 remaining progeny and also 6 other adopted normal nurslings. All 10 suckled mice were resistant to fully active virus administered 16 days later, the nurslings then being 19 days old. Five litter mates of the adopted nurslings, after nursing their nonimmune mother for 16 days, died as the result of similar amounts of virus. All of the mixed natural and adopted litter succumbed to a second injection of virus administered at three months of age. The immunity at an early age is interpreted as due to passively acquired immunity, presumably through protective antibodies contained in milk. Subsequent litters of the original nursing mother were shown to have a temporary passive immunity. These observations are in accord with the previous demonstration that the duration of maternal passive immunity in suckled offspring of immune female mice is about two months[1] and that actively acquired immunity following inapparent infection is permanent. The nursing mother in this instance had evidently suffered an inapparent infection, as the result of eating her two progeny. The more important inference was that susceptible normal mice could be passively protected through suckling the immune female, and that the immunity so conferred might be sufficient to permit active immunization with fully active virus, as with the progeny of immune mothers.

MATERIALS

Virus.—The M M virus used in these experiments has been described elsewhere[2] in relation

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1. Curley, F. J., and Gordon J. E. 1948, Immunization of young mice with unmodified M M mouse encephalomyelitis virus under passive protection from immune mothers. Am. J. Hyg. **48**: 81–86.

2. Jungeblut, C. W. 1944. Serologic relationships within the poliomyelitis group of viruses. Am. J. Pub. Health, **34**: 259–264.

to the virus of human poliomyelitis and of spontaneous mouse encephalomyelitis. The immunological position is not clearly established but is close to the virus isolated by Theiler.[3] It is recognized here as a mouse encephalomyelitis virus. In our experience, M M virus is highly pathogenic for albino mice, for rarely does a mouse survive active symptoms of the disease. Death without preceding paralysis is not uncommon, and less frequently occurs with no preceding symptoms. The test inoculum was a 10% emulsion of infected mouse brain in physiologic salt solution.

sistance to ordinarily fatal doses of M M virus, usually before 21 days but no later than the 35th day of life. Survivors received a second dose at 70 to 84 days. The data for 63 nursling mice are presented in table 1.

The initial injection at ages 8 to 35 days of normal mice suckled by immune foster mothers was usually without discernible effect. Control animals

TABLE 1.—*Protection of normal newborn mice against lethal doses of M M mouse encephalomyelitis virus through suckling immune foster mothers until 21 days of age.*

Lot	Number of mice	Age first suckled by immune females (days)	Age at first challenge test (days)	Percent surviving	Average age at second challenge test (days)	Percent surviving
1	20	1 to 5	8 to 13	100	75	10.5
2	21	1 to 5	14 to 21	100	70	17.6
3	22	1 to 4	17 to 35	77	84	40.0
Total	63			92		22.7

Animal.—The newborn mice used in this study were from an albino stock known to be free from M M strain of mouse encephalomyelitis. The normal mouse aged less than 30 days dies regularly when injected subcutaneously with 0.01 ml of virus emulsion, at ages 30 to 35 days with 0.02 ml intraperitoneally, and at older ages somewhat less regularly with intraperitoneal doses of 0.05 ml. The incubation period is somewhat longer with older mice, two or three days for young mice, and for older animals, three to nine days.

Litters of newborn mice were tested for susceptibility shortly after birth by injecting a third of their number with active unmodified virus. If all test animals died, the remaining litter mates were used in these experiments. They were permitted to nurse lactating females proven to be immune to M M virus as the result of active immunization several months previously.

EXPERIMENTAL

Passive protection of normal adopted nurslings.—Normal suckling mice born of nonimmune mothers were put to nurse immune foster mothers within 5 days after birth. They were weaned at 21 days of age. All were tested for re-

suckled by nonimmune mothers invariably died. Two mice of lot 2, suckled for 12 days, experienced a modified disease but recovered. One mouse of lot 3 died as a result of a test dose at 17 days, as did 4 others injected at 32 days of age. Some 77% of survivors failed to withstand the later second injection, indicating that the initial protection was passive and temporary.

A second similar experiment was designed to determine the promptness with which passive protection was induced (table 2). Group 1, the controls, were normal newborn mice suckled by nonimmune mothers. The 8 normal sucklings of group 2 were injected with virus immediately before being placed with immune foster mothers. All of group 1 and 6 of 8 in group 2 died as a result of the test dose. Nine other groups of 6 to 8 normal newborn mice, placed with lactating immune females when two days old, subsequently received a single dose of virus, the first group 6 hours after adoption, the next three at successive 6-hour intervals to 24 hours, and the remaining 5 at intervals of a day

3. Theiler, M. 1937, Spontaneous encephalomyelitis of mice, a new virus disease. J. Exper. Med. **65**: 705–719.

TABLE 2.—*Required time for induction of passive protection in normal newborn mice against M M mouse encephalomyelitis virus through suckling immune foster mothers.*

Group	Number of mice	Interval between adoption and test injection	Result	
			Died	Survived
1	8	Controls (suckled by non-immune)	8	0
2	8	At time of adoption	6	2
3	8	6 hours	1	7
4	8	12 hours	0	8
5	8	18 hours	0	8
6	8	24 hours	0	8
7	7	2 days	1	6
8	7	3 days	0	7
9	7	4 days	0	7
10	6	5 days	0	6
11	6	6 days	0	6

through the 6th day. One of 8 mice in the 6-hour group died and also one of 7 that had suckled for two days (group 7). All others, 63 of 65, survived through the 21-day nursing period. In all, 121 (95%) of 128 young mice suckled by immune foster mothers were protected during the nursing period against ordinarily fatal doses of M M virus. Survivors usually succumbed to second doses administered in the third month. The observed resistance was temporary and presumably passive, with little or no evidence of an active immunity.

Active immunization of mice suckled by immune foster mothers.—The 62 mice in this experiment were born of non-immune females and had been suckled from the 2nd to the 21st day of life by immune foster mothers. All had received a single dose of M M virus during the first 6 days of life without evidence of manifest disease. Half (31) were reserved as controls. The second half received three repeated intraperitoneal injections of unmodified virus at ages 50, 60, and 70 days. Test was made for a possible actively induced immunity as the result of this treatment by administering a challenge dose of virus at a later age when passive immunity is known to have ended, and further by testing the next succeeding generation born of survivors for evidence of maternally transmitted passive immunity. The control group was similarly examined.

Three mice died in the course of immunization (table 3). The others were proved to have been actively immunized, since all withstood a challenge dose at 98 days of age, while 74% of controls died. The offspring of treated mice were immune; those of control mice were not, as determined by test 3 to 5 days after birth.

These results with adopted normal mice parallel those previously reported[1] for the natural progeny of immune mothers, where some 80% of suckled mice were rendered actively immune as a result of a single injection of unmodified virus, administered during a limited period in the second month of life. The varying response of the other 20%, sometimes no disease but no protection and sometimes fatal disease, indicates differences in the extent of passive protection possessed by mice of a common age under the stated conditions. Subsequent experiments showed that repeated injections of virus, when spread

TABLE 3.—*Active immunization of mice with unmodified M M mouse encephalomyelitis virus.*

Normal mice suckled by immune foster mothers until 21 days of age	Active immunization			Challenge test for immunity			Immunity passively transmitted to offspring		
	Age when injected (days)	Result D*	S†	Age (days)	Result D	S	Age offspring tested (days)	Result D	S
Treated	50, 60, 70	3	28	98	0	28	3 to 5	3	81
Controls				98	23	8	3 to 5	100	0

* D = died.
† S = survived.

through the period of temporary protection, were more likely to result in active immunity and to cause fewer deaths. Consequently, that procedure was followed in the present experiments.

DISCUSSION

A passive protection against M M mouse encephalomyelitis virus is conferred upon adopted nonimmune nurslings by the milk of immune mice, sufficient to permit active immunization with fully active virus. The failure of virus to immunize when administered during the first month of life, and the successful result during the second month, was presumably determined by differences in the amount of persisting passive protection at those ages. The high grade protection maintained while nursing led to prompt neutralization of the virus introduced, an absence of clinical effect, and no active immunity. The expected decline in antibody titer after weaning apparently permitted, when virus was introduced during the second month, a balance between infection and resistance of such nature as to give rise to latent active infection and a resulting permanent immunity. After two months, passive protection had largely disappeared, for essentially all animals died.

The parallel experience of mice born of immune and of susceptible mothers, provided they are suckled by an immune lactating female, identifies the observed passive protection to be a function of specific protective antibodies rather than of genetic inheritance. Other experiments have demonstrated the anticipated passive protection of unsuckled progeny of immune mice, presumably through placental transmission of antibodies. The protection was shorter than when mice were suckled. The added antibodies from the blood did not prolong the period of protection that followed suckling alone.

The important consideration is that an existing host susceptibility was so modified as to produce a temporary immunity permitting active immunization with fully virulent virus. This contrasts with the conventional approach to artificial immunization, where the normal host is treated with a modified virus, altered in such fashion as to be less virulent or lack pathogenic properties, although retaining ability to immunize. The principle may well have wider application than to the host and agent studied here.

SUMMARY

Specific protective antibodies against M M strain of mouse encephalomyelitis virus, transmitted passively from immune female mice through milk to adopted nonimmune sucklings, protect against otherwise lethal doses of virus. The immunity is essentially complete during the first month of life; it ends in the third month. The balance between infection and immunity brought about by administration of unmodified virus during the second month results in latent infection and active immunization.

INDUCED MODIFICATIONS IN THE ANTIGENIC REACTIVITY OF THE PNEUMOCOCCUS

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The present study was undertaken to learn whether the antigenic patterns of pneumococci could be modified by cultivating them in heterologous immune serums containing cross-reacting antibodies. The work was prompted by the observation of such a change in a pneumococcus isolated from the cerebrospinal fluid of a patient undergoing serum therapy.

It has long been known that a type-specific pneumococcus grown in homologous antiserum may lose its capsule, type specificity, and virulence.[1–4] Similar changes have also been noted in pneumococci isolated from horses undergoing immunization with live culture.[5] Griffith[6] reported an instance in which a type I strain grown in type II serum produced rough colonies. However, a review of the literature has not revealed evidence of specific modifications caused by growth in heterologous serums which contain cross-reacting antibodies.

In 1938 a type 31 pneumoccocus isolated from the cerebrospinal fluid of a patient with penumococcus meningitis appeared to change its type specificity following the patient's treatment with type 31 rabbit serum. Both the culture isolated before serum therapy and that recovered later from the patient reacted in type 61 (Eddy[7]) serum, and, since the diagnostic serum that had been used crossed with type 61, it was postulated that the infecting microorganism was a type 61 with a type 31 cross which had been lost in consequence of the serum treatment. The present experiments were undertaken to explore this possibility. Unless otherwise indicated, the type designations are those of Cooper[8,9] and Walter.[10]

METHODS

Serial transfers of a 16-hour broth

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2. Griffith, F. 1923, The influence of immune serum on the biological properties of pneumococci. Great Britain Ministry of Health, Reports on Public Health and Medical Subjects, No. 18: 1–13.
3. Reimann, H. A. 1925, Variations in specificity and virulence of pneumococci during growth *in vitro*. J. Exper. Med. **41:** 587–600.
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8. Cooper, G., Edwards, M. and Rosenstein, C. 1929, The separation of types among the pneumococci hitherto called Group IV and the development of therapeutic antiserums for these types. J. Exper. Med. **49:** 461–474.
9. Cooper, G., Rosentsein, C., Walter, A. and Peizer, L. 1932, The further separation of types among the pneumococci hitherto included in Group IV and the development of therapeutic antisera for these types. J. Exper. Med. **55:** 531–554.
10. Walter, A. W., Guevin, V. H., Beattie, M. W., Cotler, H. Y. and Bucca, H. B. 1941, Extension of the separation of types among the pneumococci: Description of 17 types in addition to types 1 to 32 (Cooper). J. Immunol. **41:** 279–294.

culture of a single-colony fishing of a strain of one type were made in broth containing approximately 10% of serum of a related type. Neufeld and agglutination tests were made to detect changes in type-specific or heterologous reactivity. If changes were noted, the modified strain was compared with the parent for cross reactivity, reciprocal absorption, stability on animal passage, mouse virulence, and immunizing properties. Rabbits were immunized for from 6 to 8 weeks with formalin- and heat-treated vaccines.

TABLE 1.—*Serologic activity of strains and serums of types P61 and M61.*

Strains	Antiserums (types)										
	P61		M61								
	Unabsorbed	Absorbed with M61	Unabsorbed	Absorbed with P61	35A	35B	35C	31	20	40	13
Type P61	+	+	+	−	+	+	+	+	+	+	−
Type M61	+	−	+	+	+	+	+	−	−	−	+
Type 31	+	+	−	−							
Type 13	−	−	+	+							

+ denotes capsular swelling.

RESULTS

Loss of cross reactivity with retention of type specificity

Experiment I—Pneumococcus type 61 grown in type 31 serum.—Two type 61 strains were used: number 38107 isolated before serum treatment from the patient with meningitis, and a representative strain, 44193, obtained from Dr. Bernice Eddy of the National Institute of Health. The results were essentially the same with both strains, and only one experiment will be described in detail.

Transfers of the type 61 culture were made in broth containing type 31 serum which agglutinated the type 61 culture to a titer of approximately 1:60. After three transfers, the type 61 culture grew with a heavy, even turbidity; a subculture in broth alone was agglutinated by type 61 but not by type 31 serum. Thus, the type 61 culture appeared to have lost the antigenic component it had had in common with type 31. This modified strain will be referred to as type M61, the parent strain as type P61. No further change in the antigenic activity of either strain occurred during 28 mouse passages. Both strains were of low virulence, requiring at least 0.1 ml to kill a mouse; but limited tests indicated that the modified strain may have been slightly the less virulent.

When the strains were tested in unabsorbed serums of heterologous types, other differences became evident (see table 1). The loss of cross reactivity by type M61 for types 31, 20, and 40 probably represents the loss of only one common component. Reactivity for type 13 was acquired. Thus, growth in type 31 serum so modified the strain that it both lost and acquired antigenic activity not demonstrable in the parent strain.

Serums from two rabbits immunized with the parent strain and from two immunized with the modified strain were tested for specific reactivity and for cross reactivity with strains of 85 types. All serums agglutinated the parent and modified strains to a titer of approximately 1:200. Tests of the serums for capsular swelling showed that the loss or gain in the antigenic activity of the modified strain was reflected in the antibodies it produced as an immunizing agent.

TABLE 2.—*Serologic activity of strains and serums of types P31 and M31.*

Strains	Antiserums (types)												
	P31 (rabbit 1)		P31 (rabbit 2)		M31								
	Unabsorbed	Absorbed with M31	Unabsorbed	Absorbed with M31	Unabsorbed	Absorbed with P31	20	35A	35C	40	61	35	38
Type P31	+	+	+	+	+	−	+	+	+	+	+	−	−
Type M31	+	−	+	−	+	+	−	−	−	−	−	+	+
Type 20	+	+	−	−	−	−							

Table 1 shows the failure of reciprocal absorption, indicating distinct antigenic differences in the two strains. Homologous reactivity was not removed from a type P61 serum absorbed with the type M61 culture, or from a type M61 serum absorbed with type P61 culture.

Experiment II—Pneumococcus type 31 culture grown in type 20 serum.—A type 31 culture was so modified by growth in type 20 serum that it lost its cross reactivity for types 20, 35A, 35C, 40, and 61, although it retained its type 31 specificity and gained reactivity for types 35 and 38 (table 2). Virulence for mice was reduced; the minimum lethal dose was increased from 10^{-6} to 10^{-2} ml of culture. Thirty mouse passages did not alter the serologic reactivity of the parent or modified strains.

The differences in antigenic reactivity were also demonstrated in serums produced with both strains. However, only one serum produced with the parent strain reacted with cultures of types 20 and 40, apparently because of differences in the immune response in the individual rabbit. The results of absorption tests in table 2 show the failure of reciprocal absorption. That the reactivity remaining in the absorbed type P31 serum was not due entirely to antibody formed in response to the type 20 cross-reacting antigenic component is shown by the fact that the antigenic activity was demonstrated in a serum that did not cross react with type 20. In a control series this type 31 strain was transferred 7 times in broth containing type 1 serum that did not cross react with type 31. It showed no changes in antigenic activity or virulence.

Experiment III—Pneumococcus type 20 grown in type 31 serum.—A type 20 strain lost reactivity for type 31 when grown in type 31 serum. The change remained stable through 23 mouse passages. Virulence for mice was only slightly reduced; the minimum lethal dose was increased from 10^{-7} to 10^{-6} ml of culture.

Experiment IV—Pneumococcus type 40 grown in type 20 serum.—A type 40 culture grown in type 20 serum broth lost cross reactivity for type 20. Both the parent and modified strains were of low virulence, requiring 0.5 ml of culture to kill a mouse. The antigenic reactivity of the strains was not altered by 28 mouse passages. A comparison of the cross reactivities of the strains is given in table 3. The type M40 strain acquired reactivity for type 13, but to a lesser degree than did type M61. Moreover, type M40 immune rabbit serum did not cross react with type 13,

TABLE 3.—*Serologic activity of types P40, M40, and 40A strains.*

Strains	Antiserums (types)									
	P40	M40	20	31	35A	35C	61	30	39	13
Type P40	+	+	+	+	+	+	+	+	+	−
Type M40	+	+	−	−	−	−	−	+	+	+
Type 40A	+	+	−	−	−	−	−	+	+	+

indicating that this component does not readily induce the production of antibodies. Type 40A, established by Walter,[10] gave cross reactions similar to the type M40. Reciprocal absorption tests also indicated that the two strains, type 40A and M40, are identical (table 4).

Loss of type specificity

The circumstances which seemed to induce loss of type specificity suggested that the major antigenic component was shared. When a type 11A culture was grown in broth containing type 11 serum, reactivity for both type 11 and type 11A was lost. Virulence for mice was markedly reduced. The parent strain was fatal in a dose of 0.01 ml, while 1.0 ml of the degraded strain failed to kill. Similar results were obtained with several other types: type 11 grown in type 11A serum, type 22A in type 22 serum, type 11B in type 11 serum, and type 35B in type 29 serum. In all instances, the modifying serum agglutinated the homologous culture to only a slightly higher titer than the cross-reacting culture, indicating a very close relation between the strains.

TABLE 4.—*Absorption tests of serums of types P40, M40, and 40A.*

Strains	Type P40 antiserum			Type M40 antiserum			Type 40A antiserum		
	Unabsorbed	Absorbed with M40	Absorbed with 40A	Unabsorbed	Absorbed with P40	Absorbed with 40A	Unabsorbed	Absorbed with P40	Absorbed with M40
Type P40	+	+	+	+	−	−	+	−	−
Type M40	+	−	−	+	+	−	+	+	−
Type 40A	+	−	−	+	+	−	+	+	−
Type 20	+	+	+	−	−	−	−	−	−
Type 31	+	+	+	−	−	−	−	−	−
Type 35A	+	+	+	−	−	−	−	−	−
Type 35C	+	+	+	−	−	−	−	−	−
Type 61	+	+	+	−	−	−	−	−	−
Type 30	+	−	−	+	−	−	+	−	−
Type 39	+	−	−	+	−	−	+	−	−
Type 13	−	−	−	−	−	−	−	−	−

DISCUSSION

It has been shown that a pneumococcus strain grown in diluted serum produced against a heterologous cross-reacting type may lose its reactivity in both homologous and cross-reacting serums. In other cases, only cross reactivity is lost and reactivity not previously demonstrable may appear. The modifications are permanent, in that they persist through numerous transfers and mouse passages. Such changes are probably related to those that occur in transformations from the smooth to the rough form by growth in homologous immune serum.

Two explanations may be suggested. One theory, often used to explain the change from the rough to smooth form, is that the dominant parent strain is sufficiently suppressed by antibody to permit the emergence of a mutant. An alternative theory is that genes and surface antigens are similar and that antibodies may therefore directly influence the genes.[11–14] Emerson, in a discussion of gene structure, states, "The presence of the antibody molecule on the surface of the gene would so change that surface

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12. Sturtevant, A. H. 1944, Can specific mutations be induced by serological methods? Proc. Nat. Acad. Sc. **30**: 176–178.
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that the gene could no longer make an exact copy of itself, but would either fail to reproduce, or would produce a new gene with an altered surface. Either of these results would be recognizable as a mutation since the descendants of this cell could no longer elaborate the antigen in question."[14] In the present study, if the structure of the cross-reacting antigenic component is duplicated in the gene itself, the cross-reacting antibody might combine with and thus inactivate the corresponding structure of the gene. The result would be a mutant form lacking the cross-reacting antigenic component of the parent strain. Although the modifications suggest a definite change in the hereditary material of the cell, they afford no proof of the mechanism involved.

White's[15] theory that the many antigenically different strains of salmonella have originated from primitive types of more complex antigenic make-up is supported by the experiments of Edwards and Bruner,[16,17] which were somewhat similar to ours. If some of the pneumococcus types have originated by an analogous process, it would be extremely interesting to study the natural occurrence of such changes.

15. White, P. B. 1926, Further studies of the Salmonella group. Great Britain Medical Research Council, London, H. M. Stationery Office (Special report series No. 103).

CONCLUSION

Pneumococci grown in the serums of heterologous cross-reacting types may lose their cross reactivity while maintaining their type specificity or they may lose their activity for both heterologous and homologous serums.

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INCREASED MORTALITY FOLLOWING PENICILLIN THERAPY OF CHICK EMBRYOS INFECTED WITH *CANDIDA ALBICANS* VAR. *STELLATOIDEA*

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Marked changes in the bacterial flora of man consequent to the administration of penicillin[1] or streptomycin[2–4] have been described in the literature. Similarly, the sudden appearance of *Candida* in predominant numbers or in pure culture in routine cultures of material from patients receiving prophylactic antibiotic or antibiotic therapy for diagnoses such as scarlet fever, tonsillitis, diphtheria, etc., has been noted by a number of observers.[5]

Such changes are of no consequence in the majority of instances. However, spontaneous, new bacterial infections have been observed during antibiotic therapy in a few patients.[6] The exacerbation of mild thrush concomitant with penicillin therapy[5] and a postmortem diagnosis of generalized moniliasis made in this laboratory some time ago[7] following the intensive penicillin therapy of congenital lues, posed the question as to the effect of penicillin on *Candida*.

It was hypothesized that this apparent "enchancement"* may be the result of: (a) suppression of competitive bacterial flora, (b) direct effect on the fungus cell, or, (3) combinations of the above. That suppression of bacterial flora is at least related to this phenomenon is evidenced by the superiority of various selective fungus mediums for the isolation of fungi from pathological material. The purpose of the present report is to present evidence suggesting a stimulatory effect by penicillin on the fungus cell.

Candida is classified in this laboratory according to the system described by Martin et al.[8] *C. albicans*, isolated from oral thrush, and *C. albicans* var. *stellatoidea*, isolated postmortem from a

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6. Weinstein, L. 1947, The spontaneous occurrence of new bacterial infections during the course of treatment with streptomycin or penicillin. Am. J. M. Sc. **214**: 56–63.
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* The term "enhancement," as used throughout this study, is intended to express an actual numerical increase and/or the increased biological activity of *Candida*. Although the in vitro experiments to be presented suggest that suitable concentrations of penicillin produce a simple numerical increase of *Candida*, study of smears and histological sections of the chorioallantoic membranes indicate that this is not the entire explanation for the increased mortality observed in chick embryos. Increased biological activity as well as increased growth seems to be a factor in increased mortality.

8. Martin, D. S., Jones, C. P., Yao, K. F. and Lee, L. E., Jr. 1937, A practical classification of the monilias. J. Bact. **34**: 99–129.

TABLE 1.—*Effect of penicillin on* C. albicans *var.* stellatoidea *infection in chick embryos.*

Day of experiment	Treated (201 embryos)			Controls (202 embryos)			$\frac{x}{\sigma}$
	Number of deaths	Cumulative mortality	σ	Number of deaths	Cumulative mortality	σ	
1	12	5.9	1.59	0	0	—	—
2	42 (54)	26.7	3.01	23 (23)	11.2	2.23	6.80
3	13 (67)	33.0	3.32	14 (37)	18.2	2.73	3.56
4	13 (80)	39.5	3.46	9 (46)	22.7	3.00	3.66
5	10 (90)	44.5	3.87	10 (56)	27.6	3.16	3.40
6	4 (94)	47.0	3.75	8 (64)	31.6	3.88	3.08
Total	94	47.0 ±	3.75	64	31.6 ±	3.88	3.08
Adjusted total		38.2 ±	3.46		22.8 ±	3.00	3.39

case of chronic pulmonary infection, were used in these experiments. Both patients had received penicillin therapy. It may be of interest to note that although *Candida* was not the primary etiologic agent in the latter instance, this child's mother had had vaginal thrush during pregnancy, he himself had had neonatal thrush which cleared on gentian violet therapy, and an infant sibling now has neonatal thrush, all due to this same species of *Candida*. *Candida* reappeared in routine cultures of the nose, throat and bronchial secretions of this patient at two and one-half months of age, following four weeks of penicillin therapy of the primary pulmonary disease.

IN VIVO EXPERIMENTS

Chick embryos

Saline suspensions of *Candida* were prepared from 24-hour cultures on Sabouraud's agar and standardized by turbidity to contain approximately 5,000,000 cells per 1.0 ml. Ten-day chick embryos, prepared by the usual window technique, were inoculated on the chorioallantoic membrane with 0.02 ml (100,000 cells) of such suspensions. At the same time, the treated embryos also received a single dose of 500 Oxford Units (10 O.U. per ml of embryo) of crystalline penicillin G in 0.5 ml of saline on the chorioallantoic membrane. In order to rule out any effect attributable to this amount of fluid, the same volume of saline was added to several groups of eggs in the control series. The eggs were closed with paraffined cover slips, incubated at 35 C and examined daily for viability and evidence of infection for a period of 6 days. The eggs were at first smeared and subcultured on the first day to determine the existence of infection and the absence of contaminants.

The mortality data obtained from a series of small experimental groups run over a period of two months are sum-

TABLE 2.—*Effect of penicillin on* C. albicans *infection in chick embryos.*

Day of experiment	Treated (76 embryos)			Controls (74 embryos)			$\frac{x}{\sigma}$
	Number of deaths	Cumulative mortality	σ	Number of deaths	Cumulative mortality	σ	
1	13	7.1	2.95	1	1.3	1.03	1.83
2	20 (33)	43.5	5.66	8 (9)	12.2	3.61	4.65
3	5 (38)	50.0	5.57	13 (22)	29.5	5.29	2.70
4	5 (43)	56.8	5.74	8 (30)	40.5	5.74	2.00
5	2 (45)	59.5	5.66	12 (42)	57.0	5.74	0.31
6	4 (49)	64.5	5.48	4 (46)	62.0	5.66	0.32
Total	49	64.5 ±	5.48	46	62.0 ±	5.66	0.32
Adjusted total		55.7 ±	6.00		53.2 ±	6.08	0.29

marized in table 1. The penicillin-treated embryos started to die within the first day following inoculation, the majority of deaths having occurred at the end of the second day. The untreated controls did not attain a comparable mortality until the fifth day of incubation.

Statistical analyses* indicate a significantly greater mortality among the penicillin-treated embryos, as judged by comparison of the daily cumulative or the total cumulative mortality rates. The statistical difference is not affected when the rates are adjusted for possible traumatic deaths, an incidence of 8.8% having been observed in a comparable series of control chick embryos in this laboratory.

A smaller series of identical experiments done with *C. albicans* gave similar but less well defined results (table 2). The penicillin-treated embryos began to die within the first day following inoculation, the majority of deaths again having occurred at the end of the second day. A comparable mortality rate was not attained in the controls until the fourth day of incubation.

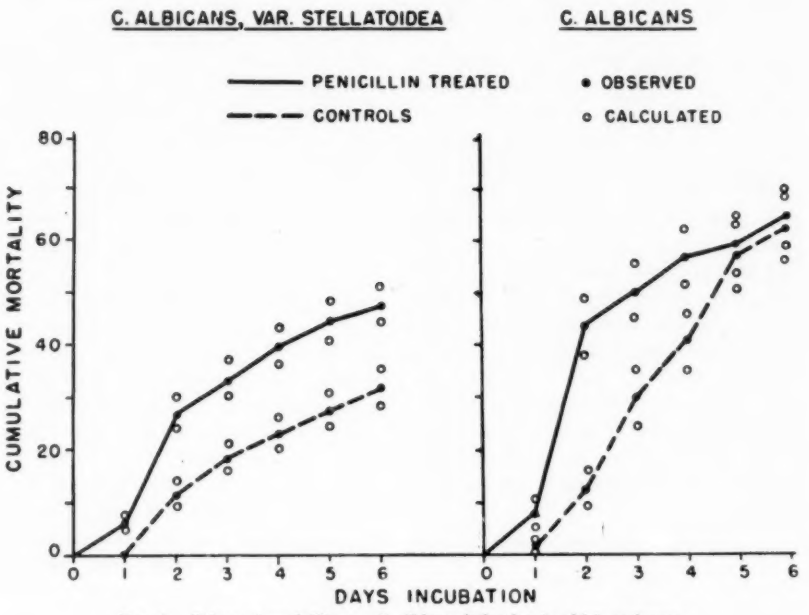

FIG. 1.—Effect of penicillin on *C. albicans* infection in chick embryos.

These mortality data are plotted in figure 1, together with the standard errors calculated for each of the observed rates. Mortality in the penicillin-treated groups began and proceeded at a constantly higher rate, the difference representing an increased mortality superimposed upon the natural mortality resulting from candida infection in the chick embryo. The similarity of the late and total cumulative mortality observed with *C. albicans* probably is a reflection of the greater natural infectivity of this strain than that of

* Computed from the formula: $\sqrt{(p \times q)/n}$. Significance is assumed where X/σ is more than 2.6.

TABLE 3.—*Effect of crystalline penicillin G on in vitro growth of* C. albicans *var.* stellatoidea

Inoculum—incubation	Oxford units per ml				
	0.01	0.1	1.0	10.0	None
Undiluted (±450 M cells)	Turbidity				
24 hr.		76.0			80.0
96 hr.		48.0			53.0
120 hr.		46.0			51.0
1:10 (±45 M cells)					
24 hr.	88.0			85.0	90.0
96 hr.	76.0			74.0	82.0
120 hr.	71.0			69.0	77.0
1:10,000 (±0.045 M cells)					
24 hr.		92.0	88.0		96.0
96 hr.		84.0	75.0		93.0
120 hr.		76.0	66.0		88.0

C. albicans var. *stellatoidea*. Although the mortality in chick embryos observed with this strain was less than that reported for *C. albicans* in a very small series,[9] it was significantly greater than that observed with *C. albicans* var. *stellatoidea* in both the penicillin and control embryos.

During a portion of these series of experiments, parallel serial egg passage was done with *Candida* from penicillin-treated and control chick embryos. No alteration in the respective mortality rates was noted.

No gross or microscopic difference in the pathologic lesions was observed quantitatively or qualitatively between the treated and control embryos, nor between *C. albicans* and *C. albicans* var. *stellatoidea* infections. The gross lesions were first evident as punctate white areas grouped in the region of inoculation, often most prominent along any lines of minor trauma which may have occurred consequent to the windowing procedure. By 48 to 72 hours these had become confluent to form a raised, somewhat umbilicated white plaque 1 to 2 cm in diameter, similar in appearance to human oral thrush. Subsequent to this there was an apparent contraction of the underlying membrane with a heaping-up but no further extension of the lesion.

Membranes fixed in picric acid-formalin and stained by the Giemsa method showed this heaped-up lesion to consist of a matted mass of pseudohyphae and blastospores. At the central portion of the lesion the membrane was often necrotic with little thickening. Peripherally the epithelial layers were slightly thickened without apparent pearl formation. The intervening mesoderm was edematous with moderate infiltration by mononuclear cells, and in some areas considerable increase in the number and density of the connective tissue cells. Pseudohyphae were seen growing into and through the mesoderm, in some instances producing free arborizations into the allantoic space. No blood vessel invasion was observed.

Coronal sections of the head and sagittal sections of the body of 24 embryos fixed and stained by the same method showed no evidence of invasion or infection save in a single instance where there was a small focus of mycelial growth in the liver unassociated with necrosis or cellular reaction. These findings are essentially similar to those recorded elsewhere.[10,11]

Rabbits

A similar enhancing effect has been observed in penicillin-treated rabbits. A pair of 2 kg rabbits were given a single intramuscular injection of 150,000 Oxford units of crystalline penicillin G. The following day these rabbits, together with two controls of the same

9. Meyer, E. and Ordal, Z. J. 1946, Pathogenicity of *Candida* species for the chick embryo. J. Bact. **52:** 615–616.

10. Moore, M. 1941, The chorioallantoic membrane of the developing chick as a medium for the cultivation and histopathologic study of pathogenic fungi. Am. J. Path. **17:** 103–120.

11. Norris, R. F., Shorey, W. K. and Bongiovanni, A. M. 1948, Lesions produced in chick embryos by *Candida* (*Monilia*) *Albicans*. Arch. Path. **45:** 506–512.

weight, were injected intravenously with 1.0 ml of a suspension of the same strain of *C. albicans* var. *stellatoidea* used in the chick embryos, prepared by suspending the growth from a 24-hour Sabouraud's agar slant in 10 ml of saline. The treated animals continued to receive a single daily intramuscular dose of 150,000 Oxford units of crystalline penicillin G.

Both of the penicillin-treated rabbits were dead 4 days after inoculation (after 5 daily doses of penicillin); one control died at 9 days, and the remaining control was sacrificed at 16 days. *C. albicans* var. *stellatoidea* was recovered at autopsy from the viscera of all animals.

The main pathologic features were the same in both penicillin-treated animals. The kidneys were pale and overweight and on section showed many 1 mm white areas filling and almost limited to the cortex. Microscopically these were miliary abscesses, mostly located in and destroying glomeruli. The centers were composed of blastospores and necrotic debris with a surrounding collar of mononuclear and a variable number of polymorphonuclear cells. The tubules appeared to be normal. In the myocardium the lesions were larger than in the penicillin-treated animals and had the same general structure as in the kidney.

The control rabbit sacrificed at 16 days showed strikingly fewer lesions than the other animals, probably a reflection of a lighter initial infection rather than of rapid healing. The kidneys showed a few inflammatory lesions with central giant cells, epithelioid cells and a few peripheral lymphocytes. The tubular lesions were more striking, consisting of radial bands of dilated tubules, some with markedly vacuolated epithelium (a late change seen in many toxic nephroses in rabbits), and a small amount of interstitial cellular reaction. The intervening tubules were unaffected. The myocardial lesions were limited to a few focal collections of lymphocytes.

These lesions are consistent with those previously described in experimental candida infection in rabbits.[12]

IN VITRO EXPERIMENTS

In contrast to previous experience with *Cryptococcus neoformans*,[13] in vitro penicillin enhancement was not readily demonstrable with *Candida*. Several series of experiments with various mediums inoculated in the conventional manner with a number of strains of *Candida* failed to show a penicillin-enhancing effect. However, when the inoculum was titrated against increasing amounts of crystalline penicillin G in a synthetic asparagine-1.0% glucose broth,[14] some evidence of in vitro enhancement was obtained.

Saline suspensions of the *C. albicans* var. *stellatoidea* used in the previous experiments were prepared from 24-hour Sabouraud's agar slants to equal the density of a barium sulfate standard #15. Serial 10-fold dilutions through 1:1,000,000 were prepared from this suspension, and 0.1 ml of each dilution was planted in a series of 10 ml samples of asparagine-1.0% glucose broth containing crystalline penicillin G in 10-fold concentrations ranging from 0.01 to 10.0 Oxford units per ml. The titrations

12. Stovall, W. D. and Pessin, S. B. 1933, Classification and pathogenicity of certain *Monilias* Am. J. Clin. Path. **3**: 347–365.
13. Foley, G. E. and Farber, S. 1948, Observations on the biology of *Cryptococcus neoformans:* correlation with the pathological changes observed in three cases of crypto coccosis in infants. Proc. 48th Gen. Meet., Soc. Am. Bact. **1**: 66–67.
14. Salvin, S. B. and Hottle, G. A. 1948, Factors influencing histoplasmin formation. J. Bact. **56**: 541–546.

were incubated at 37 C and turbidity determined at 24-hour intervals in the Evelyn photoelectric colorimeter against the 620 μ filter.

Table 3 presents the average data from three experiments in which a penicillin-enhancing effect was observed with one or more dilutions of inoculum at one or more concentrations of penicillin. The relationship between the inoculum and concentration of penicillin seems to be of critical importance in view of the various concentrations of penicillin showing enhancement effects on different inoculums. The uncontrollable variations, viz., the physiologic state and number of viable cells in the inoculum, undoubtedly have some effect on the results obtained in such experiments. Although by no means conclusive, the data suggest an in vitro enhancing effect of penicillin G on *C. albicans* var *stellatoidea*.

DISCUSSION

The in vitro enhancement of various microorganisms by penicillin, streptomycin and other antibacterial substances is a well established phenomenon and has been reviewed recently in relation to another study.[15] In vivo enhancement analogous to that reported here has been observed with *Salmonella typhi* in mice treated with patulin,[16] streptomycin[17] and penicillin [18] and with *Mycobacterium tuberculosis* in guinea pigs treated with penicillin.[19]

The mechanism of this in vivo antibiotic enhancement is not yet known. However, evidence obtained from in vitro experiments with other fungi[13] and bacteria[20] indicates that enhancement is the result of a metabolic stimulation either by direct utilization of the antibiotic itself or by the metabolites made available to survivors by the lysis of antibiotic-sensitive cells.[21]

The present in vitro experiments with *C. albicans* var. *stellatoidea*, although by no means conclusive, suggest metabolic stimulation by penicillin. The in vivo appearance of streptomycin-dependent strains of *Myco. tuberculosis*[22] and other microorganisms[3,4] indicates the possibility of direct antibiotic utilization in vivo. However, in the case of *Candida*, an increased production and release of toxins or antigens precipitating allergic phenomena similar to the Herxheimer reaction in syphilis may be related to the in vivo effect of penicillin as well. Further studies on the mechanism of enhancement are not in progress.

The phenomenon of antibiotic enhancement becomes of more than academic interest when it can be demonstrated in vivo. Its potential significance

15. Foley, G. E. and Shwachman, H. Some observations on a streptomycin-dependent strain of *Staphylococcus aureus*. In press.
16. Stansfeld, J. M., Francis, A. E. and Stuart-Harris, C. H. 1944, Laboratory and clinical trials of patulin. Lancet, **2**: 370–372.
17. Welch, H., Price, C. W. and Randall, W. A. 1946, Increase in fatality rate of *E. typhosa* for white mice by streptomycin. J. Am. Pharm. A. **35**: 155–158.
18. Randall, W. A., Price, C. W. and Welch, H. 1947, Demonstration of hormesis (increase in fatality rate) by penicillin. Am. J. Pub. Health, **37**: 421–425.
19. Riviere, C., Thely, M. and Gautron, G. 1947, Action accélératrice exercée en de certaines conditions, par la pénicilline sur l'évolution de la tuberculose experimentale du cobaye. Compt. rend. Acad. d. sc. **224**: 1856–1857.
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21. Eriksen, K. R. 1946, Some studies on the lytic action of penicillin on *Staphylococci* and *Pneumococci*. Acta. path. et microbiol. Scandinav. **23**: 221–228.
22. Lenert, T. F. and Hobby, G. L. 1949, Streptomycin-dependent strains of *M. tuberculosis*. Am. Rev. Tuberc. **59**: 219–220.

with a microorganism as ubiquitous as *Candida* is obvious, especially in early life where *Candida* carriage or mild infection is not uncommon.

SUMMARY

The administration of a single dose of 500 Oxford units of crystalline penicillin G on the chorioallantoic membrane of 10-day chick embryos increased the mortality resulting from *Candida albicans* var. *stellatoidea* or *C. albicans* infection.

A similar enhancing effect was observed in rabbits infected with *C. albicans* var. *stellatoidea* and treated with a daily intramuscular dose of 150,000 Oxford units of crystalline penicillin G.

Preliminary observations further suggest that penicillin also has an in vitro stimulatory effect on *C. albicans* var. *stellatoidea*.

VIRUSES OF THE LYMPHOGRANULOMA-PSITTACOSIS GROUP ISOLATED FROM OPOSSUMS IN COLOMBIA. OPOSSUM VIRUS A

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In the latter part of the year 1943 ten opossums in the animal house of the Carlos Finlay Institute were affected by a disease of which the principal symptom was paralysis of the hind quarters and which caused death after three or four days.

In the course of the investigation of the agent of this epidemic two strains of a virus with all the characteristics of the viruses of the lymphogranuloma-psittacosis group were isolated. The virus produced a meningitis in the albino Swiss mouse when injected intracerebrally and a pneumonitis when given intranasally. The pathological findings in mice infected with the virus showed lesions which were similar to those produced by the meningopneumonitis virus isolated by Francis and Magill[1] from ferrets. The newly isolated virus has been tentatively called opossum virus A.

Another virus of this group, isolated from a Colombian marsupial, which will be described in a later paper, has been called opossum virus B.

Isolation of the virus

The first strain of opossum virus A was isolated from a *Didelphis paraguayensis* trapped in a district of the municipality of Cáqueza, State of Cundinamarca, Colombia. This area is situated on the eastern side of the Andes. After 45 days of captivity, the animal became sick, exhibiting paralysis of the hind legs and convulsions. It was bled from the heart, and two groups of adult mice were inoculated intracerebrally with its serum. On the next day the opossum was found dead, and two other groups of mice were inoculated with a 10% suspension of its brain in saline solution. The autopsy of the animal showed only some congestion of the brain and an extensive hemorrhage into the thoracic cavity resulting from the heart puncture of the day before. However, microscopic examination of brain sections showed a meningitis.

One mouse of the groups injected with the serum of the opossum showed hypersensitivity and roughening of the coat on the 7th day after inoculation. This mouse was sacrificed, and a 10% suspension of its brain was prepared in saline solution. After the suspension was centrifuged, the supernatant was injected intracerebrally into a group of adult mice. From that group of mice serial passages of the virus by the intracerebral route were established.

The mice injected with the suspension of the opossum's brain did not show any symptoms of illness during an observation period of one month.

The second strain of opossum virus A was isolated from an animal of the species *Caluromys laniger* six months after the isolation of the first strain. This animal was trapped in the Magda-

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1. Francis, T., Jr. and Magill, T. P. 1938, An unidentified virus producing acute meningitis and penumonitis in experimental animals. J. Exper. Med. **68**: 147–160.

lena Valley, on the west slope of the Andes. Three weeks later it became sick. There were some oppossums from the Cáqueza district in the animal house at that time, but none of them showed any signs of illness.

The caluromys had the same symptoms as the opossum from which the first strain of the virus was isolated. The animal was sacrificed, and a 10% suspension of a pool of liver, spleen and kidney was prepared in saline solution. After this was centrifuged at 1500 r.p.m., the supernatant was injected intracerebrally into two groups of adult mice. All these mice were sick from the 5th to the 7th day after inoculation; they had the same symptoms as the mice infected intracerebrally with the strain of virus from the didelphis established in adult mice. Serial passages by the intracerebral route were established from these groups of mice infected with the caluromys material. The two strains of opossum virus A have been called the didelphis strain and the caluromys strain respectively.

Properties of the virus

Repeated cultures from brain suspensions used in the serial passages, made in solid and liquid medium under aerobic and anaerobic conditions, failed to reveal any bacteria which could reproduce the experimental disease. On the other hand, impression smears prepared from brains and lungs of sick mice infected with the virus showed minute granules of reddish-purple color when stained with either Giemsa or Wright stain. When stained by the Macchiavello method they took the basic fuchsin color, and with the Ruiz-Castañeda technique they took the methylene blue color. On the basis of their characteristics, size and the fact that they are to be found intra- and extracellularly, these granules resemble the elementary bodies of the viruses of the lymphogranuloma-psittacosis group.

Opossum virus A, like other agents of the lymphogranuloma-psittacosis group inoculated into the yolk sac, grew easily in the developing chick embryo. The virus multiplied in the yolk sac cells, producing an endotoxin which killed mice when heavily infected yolk sac suspensions were injected intravenously.

The virus was cultivated without difficulty in tissue culture, using chick and mouse embryos in Tyrode solution with 10% human serum.

The virus passed with comparative ease through a Berkefeld V filter, but did not pass a Berkefeld N nor a Seitz filter. The filtrates induced typical, although delayed, infection in mice injected intracerebrally.

The virus was preserved by desiccation for a considerable time. A 10% mouse brain suspension in rhesus monkey serum, desiccated from the frozen state, was found to produce typical infection in mice when it was injected intracerebrally into these animals more than one year later. Similarly 5% suspension of yolk sacs in broth, from cultures of the virus in chick embryos, produced typical infection in mice after 10 months in the desiccated state.

Pathogenicity of the virus for various animals

Albino Swiss mouse.—The virus transferred to mice by intracerebral inoculation of the whole blood or suspensions of liver, spleen and kidney from infected opossums needed very little adaptation to become established in serial passages, producing then the typical paralysis of the hind legs and death of the animals in 4 to 6 days. The symptoms presented were very similar to those shown by mice infected by the same route with the meningopneumonitis virus described by Francis and Magill.[1] In the

mice showing typical paralysis, the brain was swollen and edematous, and the characteristic lesions of a generalized meningitis were seen in the microscopic examination of the brain sections.

As a result of the serial passages, the virulence of the virus was increased, and after 100 passages the 50% mortality end point[2] occurred in the 1:85,000 dilution. Following infections after inoculation of serial 10-fold dilutions of brain suspensions, the incubation period depended on the concentration of the virus, though mice rarely showed symptoms after the 10th day. The more typical symptoms of the disease were observed in the animals infected with smaller doses. Occasionally mice recovered but maintained paralysis of indefinite duration.

When mice were inoculated by the intranasal route, an entirely different disease was produced. No neurological signs were apparent, but the infected mice showed marked symptoms characteristic of lung infection, i.e., respiratory distress with audible wet respirations. The incubation period depended upon the concentration of the virus inoculated. After serial passages were established, death occurred on the second to the fourth day following the inoculation of 0.05 ml of a 10% suspension of infected mouse lungs. In mice infected with smaller doses, death was delayed for as long as 6 to 10 days; occasionally the animals showed symptoms after the 10th day.

The lungs of mice infected by the intranasal route showed dark red pulmonary consolidation, with a cartilaginous consistency when the lungs were cut and considerable exuded edematous fluid. The lesions seen in sections of the affected lungs were indistinguishable from those produced in mice infected intranasally with the meningopneumonitis virus.

Mice from 3 to 6 weeks old, inoculated intraperitoneally with large doses of virus (from 0.5 to 1.0 ml of 10% mouse-brain suspension), showed no evidence of infection even when they were injected intracerebrally with starch solution at the same time that the virus was given. But intraperitoneal inoculation rendered the mice immune, and they survived a challenging inoculation of the virus when this was given by the intracerebral route but not when it was given intranasally.

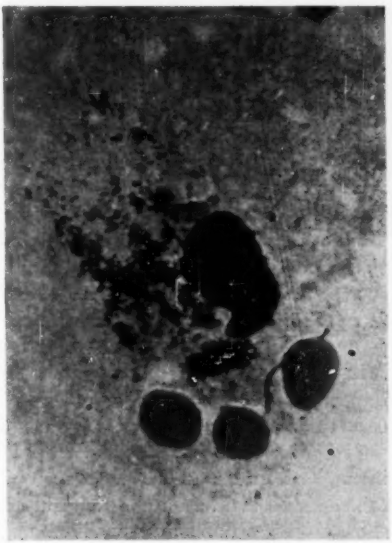

FIG. 1.—Elementary bodies in an impression preparation from a cut section of infected mouse brain (intracerebral inoculation). Giemsa stain; ×2500.

In order to demonstrate the distribution of the virus, blood was obtained from the hearts of mice used in both the intracerebral and intranasal serial passages and inoculated intracerebrally into three-week-old mice. The organs from

2. Reed, L. J. and Muench, H. 1938, A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**: 493–497.

TABLE 1.—*Distribution of the opossum virus A in adult mice infected intracerebrally.*

Mouse group no.	Strain of virus used	Days after inoculation	Results of inoculation of				
			Blood	Liver	Spleen	Kidney	Lung
K.3343	Didelphis	5	3/5*	5/5	3/4	1/4	
K.3487	Didelphis	5		5/5	4/4	3/3	4/4
K.3499	Caluromys	5		3/5	4/4	3/3	3/3
K.3503	Didelphis	5		4/4	4/4	3/4	4/4
K.3733	Caluromys	6	5/5	1/2	5/5	3/4	2/2
K.3734	Didelphis	5	3/5	5/5	3/3	3/3	1/1
K.3739	Didelphis	5	5/5	4/5	4/4	4/4	1/1
K.3767	Caluromys	3	4/5	4/4	5/5	2/2	2/2

* The numerator represents the number of mice that succumbed to the infection and the denominator the number of mice used in the test.

the animals sacrificed were separately removed, and inoculations prepared from pools of the corresponding organs were injected intracerebrally into three-week-old mice. When one of the inoculated mice showed symptoms of infection, the animal was sacrificed and a suspension of its brain was inoculated into adult mice by the intracerebral route. The distribution of opossum virus A in the blood, liver, spleen, kidney and lung of adult mice inoculated intracerebrally and in the blood, brain and pool of liver and spleen of adult mice inoculated intranasally is shown in tables 1 and 2. The mice which did not show any symptoms and survived after 15 days were challenged intracerebrally with the homologous strain (table 2). In addition, impression smears were made from the brain and stained by Macchiavello's method.

Tests for latency of infection were made in three-week-old mice inoculated intraperitoneally with 1.0 ml of 10% brain suspension and in mice surviving intracerebral passages 10 or more days after inoculation. Virus was recovered from the brains of intraperitoneally inoculated mice 15 days after inoculation, from pooled livers and spleens 21 days after inoculation and from lungs 12 days after inoculation. After infection by intracerebral inoculation, virus was recovered from brains, from pooled livers and spleens and from lungs of mice 21 days after inoculation.

Confirmation of the presence of virus was made by demonstration of the typical elementary bodies in smears stained by Macchiavello's method, prepared from brains of mice showing symptoms, and by intracerebral passages to new groups of mice. The surviving mice were tested for immunity by intracerebral challenge inoculation with the homologous strain.

Marsupials.—Pathogenicity of the virus for marsupials was tested in 13 animals, 2 *Caluromys laniger* (woolly opossum), 4 *Metachirus nudicaudatus* (brown masked opossum), 3 *Metachirops opossum* (grey masked opossum), 2 *Didelphis marsupialis* (common

TABLE 2.—*Distribution of the opossum virus A in adult mice infected intranasally.*

Mouse group no.	Strain of virus used	Days after inoculation	Results of inoculation of					
			Blood		Brain		Pool of liver and spleen	
K.3978	Caluromys	3	5/5*		0/4	(1/4)	4/4	
K.3983	Caluromys	2	5/5		5/5		5/5	
K.3991	Didelphis	3	0/5	(2/5)	0/4	(0/4)	5/5	
K.4015	Caluromys	2	4/5	(0/1)	4/4		5/5	
K.4019	Caluromys	2	4/4		3/3		4/5	(0/1)
K.4026	Didelphis	2	4/4		4/4		2/5	(0/3)
K.4048	Caluromys	2	4/4		0/5	(5/5)	3/5	(0/2)
K.4061	Caluromys	2	4/5	(0/1)	0/2	(2/2)	3/5	(0/2)
K.4073	Didelphis	2	5/5		0/4	(3/4)	4/5	(0/1)

* The numerator represents the number of mice that succumbed to the infection and the denominator the number of mice used in the test. The figures in parenthesis give the results of the challenging inoculation.

opossum) and 2 *Didelphis paraguayensis*. The specimens of *Didelphis paraguayensis* were trapped in the vicinity of Bogotá (Monserrate Hill). All the other animals were obtained from the Villavicencio (Meta) area; these latter species have been discussed by Bates.[3]

Twelve were inoculated intraperitoneally and the other subcutaneously. Eight were inoculated with mouse brain suspensions, three with suspensions of organs from infected opossums, and two with peritoneal exudate from these animals. The presence of circulating virus was investigated by bleeding the animals from the heart or from the femoral vessels and inoculating three-week-old mice intracerebrally with the blood diluted 1:2 in saline solution. Confirmation of the presence of the virus was made by intracerebral passages into adult mice and by demonstration of elementary bodies in smears prepared from the brain and stained by Macchiavello's method. Immunity was investigated in the surviving mice by challenging them intracerebrally with the homologous strain of virus used in the experiment (table 3).

The first caluromys (A.3615) became sick on the 6th day after inoculation, showing loss of appetite, marked weakness, and convulsions; it died on the 9th day. The autopsy revealed a large amount of a creamy viscous exudate in the peritoneal cavity, enlargement of the liver and spleen, and pulmonary consolidation involving the upper lobe on each side. The virus was recovered from the blood and from a pool of liver, spleen, kidney and omentum, and also from the peritoneal exudate. Elementary bodies were demonstrated in smears prepared from omentum, liver and spleen. An attempt was made to culture microorganisms from the exudate in broth and gelatin, but none were found. In the microscopic study of sections prepared from the organs, the liver showed a focal necrosis and a hyaline degeneration around the vessels, accompanied by leucocytes. The spleen exhibited extensive necrosis with hemorrhagic areas. The lungs showed edema and many capillary vessels with fibrin thrombi.

The second caluromys (A.3636), inoculated subcutaneously, did not show any symptoms and no virus was recovered from the blood, but it was immune to subsequent intraperitoneal inoculation with infectious material (mouse brain suspension).

The four metachirus studied in this experiment showed more or less the same symptoms as caluromys A.3615 (see above). The findings at autopsy were much the same. Three metachirus (A.3625, A.3627 and A.3631) showed a cerebral meningitis.

Pigeon.—Nine pigeons were tested for susceptibility to opossum virus A. Six were inoculated with 1.0 ml intramuscularly and the other three with 0.05 ml intracerebrally of the 10% mouse-brain suspension used in the routine intracerebral passages.

All the birds were bled from the 3rd to the 7th day after inoculation to determine whether virus was present in the blood stream. It was possible to recover traces of virus in only one bird (on the 6th day), which had been inoculated intramuscularly. None of the birds showed symptoms of illness during 20 to 30 days of observation.

Three birds, two inoculated intramuscularly and one intracerebrally, were sacrificed 22 days after inoculation to test possible latency of infection. Separate 10% suspensions in saline were prepared from the brain and liver of

3. Bates, M. 1944, Experiments with the virus of yellow fever in marsupials, with special reference to the brown and grey masked opossums. Am. J. Trop. Med. **24**: 91–103.

TABLE 3.—*Inoculation of marsupials with the opossum virus A.*

Animal no.	Strain of virus used	Virus preparation and dosage*	Results of tests for circulating virus† (days after inoculation)							Showing symptoms	Death or survival in days‡	
			2	3	4	5	6	7	9			
Caluromys A.3615	Caluromys mouse 151st I.C. passage	3.0 ml of 10% mouse brain susp.							6/6	Yes	D	9
Metachirops A.3624	Caluromys serum A. 3615 mouse 9th I.C. passage	3.0 ml of 10% mouse brain susp.			2/4 (0/2)	5/5	2/4 (0/2)			No	S	60
Metachirus A.3625	Caluromys serum A. 3615 mouse 11th I.C. passage	3.0 ml of 10% mouse brain susp.			5/5					Yes	D	4
D. marsupialis A.3626	Didelphis mouse 106th I.C. passage	3.0 ml of 10% mouse brain susp.			0/4 (4/4)		0/2 (2/2)			No	S	60
Metachirus A.3627	Caluromys	3.0 ml of peritoneal exudate from A. 3625	4/4	5/5	5/5					Yes	D	4
D. paraguayensis A.3628	Didelphis mouse 106th I.C. passage	2.5 ml of 10% mouse brain susp.			0/4 (2/4)	3/5 (0/2)	4/5 (0/1)	5/5		No	S	60
Metachirus A.3629	Didelphis mouse 106th I.C. passage	2.5 ml of 10% mouse brain susp.			5/5	4/4	5/5	3/3	6/6	Yes	D	9
Metachirops A.3630	Caluromys mouse 164th I.C. passage	1.5 ml of 10% mouse brain susp.			0/5 (5/5)	0/5 (5/5)	0/5 (5/5)	4/4		No	S	60
Metachirus A.3631	Caluromys mouse 164th I.C. passage	1.5 ml of 10% mouse brain susp.	4/5	5/5	5/5	5/5				Yes	D	5
Metachirops A.3632	Caluromys	2.0 ml of 10% pool of liver and kidney, A. 3627	0/2 (2/2)	4/6 (0/2)	1/4 (2/3)	5/5				No	S	60
D. marsupialis A.3633	Caluromys	2.0 ml of 10% pool of liver and kidney, A. 3627			0/5 (5/5)	0/6 (6/6)	0/5 (5/5)	0/6 (6/6)		No	S	60
D. paraguayensis A.3634	Caluromys	2.0 ml of 10% pool of liver and kidney, A. 3627		5/5	5/5					Yes§	D	4
Caluromys A.3636	Caluromys	1.0 ml of peritoneal exudate from A. 3627			0/4 (3/4)	0/6 (4/4)	0/6 (6/6)	0/3 (3/3)		No	S	60

I.C. =intracerebral.
D =death.
S =survival.
* All the animals were inoculated intraperitoneally with the exception of Caluromys A. 3636, which received the virus subcutaneously.
† The numerator represents the number of mice that succumbed to the infection and the denominator the number of mice used in the test. The figures in parenthesis give the results of the challenging inoculation.
‡ All the animals which died showed liver necrosis with the exception of *D. paraguayensis* A. 3634.
§ The autopsy showed intestinal perforation resulting from the intraperitoneal inoculation of the virus.

each bird, and each suspension was inoculated intracerebrally into 5 three-week-old mice. No virus was recovered from any of the suspensions nor did the mice show any resistance when challenged intracerebrally with the homologous strain of virus.

Guinea pig.—Four guinea pigs were inoculated with the didelphis strain (didelphis-mouse 315th intracerebral passage), three of these intraperitoneally with 3.0 ml of 10% mouse-brain suspension and one intracerebrally with 0.25 ml of the same material. Three other animals were inoculated with the same amounts of the caluromys strain (caluromys mouse 107th intracerebral passage), two intraperitoneally and one intracerebrally. The two animals inoculated intracerebrally had temperatures ranging from 39.0 to 40.2 C on the first three days. No other symptoms were noticed, and no virus was recovered from the blood stream from the 3rd to the 7th day after inoculation.

Monkey.—One rhesus, immune to yellow fever, was inoculated subcutaneously with 3.0 ml of mouse-brain suspension of opossum virus A. No symptoms were observed in the rhesus during one month of observation, and it was not possible to recover virus from its blood stream.

A similar negative result was obtained with an *Aotus laniger* (woolly monkey), which was inoculated intraperitoneally with 3.0 ml of mouse-brain suspension of the virus.

Cultivation of the virus in the chick embryo

Passages of opossum virus A in chick embryo were initiated by the inoculation of 0.5 ml of 10% mouse-brain suspension into the yolk sac of 7- to 9-day-old embryos by the Cox technique.[4] After the virus had become established through yolk sac passages, the death of the embryo occurred in 3 to 9 days after inoculation, according to the concentration of virus used. Smears prepared from infected yolk sacs showed a large number of elementary bodies. Titration of infected yolk sacs by intracerebral inoculation in adult mice revealed a high concentration of virus, with a 50% end point[2] beyond the 10^{-7} dilution.

A toxin similar to those demonstrated by Rake and Jones[5] and Hamre and Rake[6] in yolk sacs infected with the viruses of lymphogranuloma venereum, meningopneumonitis, mouse pneumonitis and feline pneumonitis was demonstrated by means of intravenous inoculation into mice weighing 12 to 14 g of 0.5 ml of inoculum prepared from heavily infected yolk sacs. The time of death varied, of course, with the degree of dilution of the inoculum. Deaths occurred as early as three hours after inoculation with a 1:20 dilution. When greater dilutions were used deaths took place from 6 to 48 hours after inoculation and on rare occasions after more than 48 hours. Forty-five percent of the deaths took place within 24 hours.

Cultivation of the virus in tissue culture

Attempts were made to cultivate the virus in tissue culture using minced chick embryo 7 days old, minced mouse embryo or minced mouse brain in Tyrode solution, with the addition of

4. Cox, H. R. 1938, Use of yolk sac of developing chick embryo as the medium for growing rickettsiae of Rocky Mountain spotted fever and typhus groups. Pub. Health Rep. **53**: 2241–2247.
5. Rake, G. and Jones, H. 1944, Studies on lymphogranuloma venereum.—The association of specific toxins with agents of the lymphogranuloma-psittacosis group. J. Exper. Med. **79**: 463–486.
6. Hamre, D. M. and Rake, G. 1944, Feline pneumonitis (Barker), a new member of the lymphogranuloma-psittacosis group of agents. J. Infect. Dis. **74**: 206–211.

10% human blood serum. The technique employed was the same as that used for the cultivation of yellow fever virus.[7]

It was possible to maintain the virus without difficulty for more than 10 serial passages through mediums prepared with chick and mouse embryos. In several attempts it was not possible to cultivate the virus in medium prepared with mouse brain tissue.

The virus showed no changes in pathogenicity for white mice after the tissue culture passages.

Cross immunity test with the didelphis and caluromys strains

During the course of this study it was possible to keep the didelphis and caluromys strains separate without contamination. In all the experimental work the behavior of the two strains was the same; a cross immunity test was therefore made to confirm their identity.

As was pointed out before in the discussion of the susceptibility of white mice to the virus, the intraperitoneal inoculation of large doses of virus produced a solid immunity to subsequent intracerebral inoculation.

Three-week-old mice were immunized against either the caluromys or the didelphis strain by the intraperitoneal inoculation of 1.0 ml of 10% mouse-brain suspension from the material used in the routine intracerebral passages. From 21 to 25 days after the intraperitoneal inoculation the mice received intracerebral challenges of a 10% brain suspension from mice used in the intracerebral passages. Mice immunized with the caluromys strain survived challenging inoculations with both the homologous (62 mice) and the didelphis strain (40 mice); and mice immunized with the didelphis strain likewise resisted both challenges (43 with the homologous strain, and 47 with the caluromys strain). Normal mice used as controls, 23 of which were inoculated with the caluromys strain and 24 with the didelphis strain, succumbed in each case.

Effect of sulfonamides on infection with the virus

The method described by Eaton and Hanford[8] was used in testing the action of sulfonamides. Sulfapyridine and sulfamerazine were each suspended in 10% gum acacia solution, and mice were given a daily intraperitoneal inoculation over a period of 4 days. The animals received in each inoculation 1.0 mg of sulfapyridine or 1.2 mg of sulfamerazine per gram of body weight. The virus was inoculated 3 to 4 hours after the first dose of drug.

In the experimental infection of mice inoculated intracerebrally, brain suspension of paralyzed mice from the routine intracerebral passages was used as source of virus in dilutions of 10^{-1} to 10^{-5}. For the mice infected by the intranasal route, suspensions of lung from mice showing typical macroscopic lung lesions from the serial intranasal passages of the virus were used as inoculum in dilutions of 10^{-1} to 10^{-4}.

The control mice were infected at the same time as the mice inoculated with the drug but received 0.5 ml of saline solution intraperitoneally.

No inhibition of the infection took place as a result of the inoculation of either drug.

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7. Lloyd, W., Theiler, M. and Ricci, N. I. 1936, Modification of the virulence of yellow fever virus by cultivation in tissues in vitro. Tr. Roy. Soc. Trop. Med. & Hyg. **29**: 481–529.

8. Eaton, M. D. and Hanford, V. L. 1945, Effect of the host in action of sulfonamides on elementary-body agents of murine and feline pneumonitis. Proc. Soc. Exper. Biol. & Med. **59**: 63–66.

Comparative study of meningopneumonitis virus and opossum virus A

In view of the fact that the behavior of opossum virus A in the albino Swiss mouse is comparable in many respects to that of meningopneumonitis virus, it was considered of importance to make cross immunity tests with the two viruses, using pigeons and mice, and at the same time to study the pathogenicity of the meningopneumonitis virus for opossums, as well as to run neutralization tests.

It was possible to obtain, through the courtesy of Dr. César Gómez of the Instituto Nacional de Higiene "Samper-Martínez," some preserved meningopneumonitis virus (strain F97-27A-M150-E3-Alantoic Fl.) which he had brought to Colombia from the Virus Laboratory of the School of Public Health, University of Michigan, where he had received it from Dr. Thomas Francis, Jr. The lyophilized material was rehydrated and injected intracerebrally into adult white mice, and serial passages were established.

Pathogenicity of meningopneumonitis virus for opossums

In the investigation of the pathogenicity of meningopneumonitis virus for opossums all the animals were inoculated intraperitoneally with suspension of brain from mice infected with meningopneumonitis virus from the routine intracerebral passages established in this institute. The technique used in tests for the presence of circulating virus was the same as that employed in investigation of the susceptibility of various animals to opossum virus A. The details of the investigation are summarized in table 4.

TABLE 4.—*Inoculation of opossums with meningopneumonitis virus**

Animal no.	Strain of virus used	Results of tests for circulating virus (days after inoculation)						Showing symptoms	Death or survival in days†
		3	4	5	6	7	8		
Metachirus A.3705	MP.F.97-27A M 150-E.3 (Bog.M.15‡)	5/5§	3/5 (0/1)	1/5 (3/3)	2/5 (3/3)	3/5 (2/2)	0/4 (3/4)	Mild	D 11‖
D. paraguayensis A.3706	MP.F.97-27A M 150-E.3 (Bog.M.15‡)	3/6 (0/2)	0/5 (5/5)	0/5 (5/5)				No	D 5¶
D. marsupialis A.3707	MP.F.97-27A M 150-E.3 (Bog.M.15‡)	0/5 (5/5)	0/4 (3/3)	0/5 (5/5)	0/4 (4/4)	0/5 (5/5)	0/5 (5/5)	No	S 60
D. paraguayensis A.3746	MP.F.97-27A M 150-E.3-(Bog.M.27‡)	0/5 (4/4)	1/5 (4/4)	0/5 (5/5)	0/5 (5/5)	0/5 (4/4)	0/5 (5/5)	No	S 60
D. paraguayensis A.3747	MP.F.97-27A M 150-E.3-(Bog.M.27‡)	1/5 (4/4)	0/5 (5/5)	0/5 (5/5)	0/5 (5/5)	1/5 (4/4)	0/5 (5/5)	No	S 60
Caluromys A.3748	MP.F.97-27A M 150-E.3-(Bog.M.27‡)	0/5 (5/5)	0/5 (4/4)	0/5 (5/5)	0/5 (5/5)	0/5 (5/5)	0/5 (5/5)	No	S 60
Caluromys A.3803	MP.F.97-27A M 150-E.3-(Bog.M.71‡)	0/5 (4/5)	0/4 (3/3)	0/5 (4/4)	0/3 (3/3)	1/5 (3/3)		No	S 60

* All the animals were inoculated intraperitoneally with 2.0 ml of 10% mouse-brain suspension.
† D = death; S = survival.
‡ Bogotá intracerebral passage in mice.
§ The numerator represents the number of mice that succumbed to the infection and the denominator the number of mice used in the tests. The figures in parenthesis give the results of the challenging inoculation.
‖ It was not possible to recover virus from any of the organ suspensions inoculated intracerebrally into mice.
¶ Autopsy showed a profuse hemorrhage into the thoracic cavity resulting from heart puncture. Neither macroscopic nor microscopic lesions were found in the organs. Traces of virus recovered from the suspension of the pool of liver, spleen and kidney.

The metachirus tested (A.3705) was more susceptible to the virus than the other marsupials. Virus was demonstrated in its blood stream from the 3rd to the 7th day after inoculation, and it showed such symptoms as loss of appetite and restlessness. It died on the 11th day after inoculation, and the autopsy revealed pulmonary consolidations involving large areas on both sides. No macroscopic lesions were seen in the other organs. From the study of sections of the organs it was found that the cause of death was a common pneumonia. It was not possible to recover virus by the intracerebral inoculation of mice with suspensions of any of the organs.

TABLE 5.—*Cross immunity tests in mice with opossum A and meningopneumonitis viruses.*

Immunizing agent	Challenging virus																	
	Meningopneumonitis dilution						Caluromys strain of opossum virus A dilution						Didelphis strain of opossum virus A dilution					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Meningo-pneumonitis virus	3/46*	1/48					1/46						3/41					
Caluromys strain of opossum virus A	10/10	10/10	10/10	10/10	8/9		0/10						0/11					
Didelphis strain of opossum virus A	27/27	28/28	27/27	27/27	28/30		0/29						0/30					
Controls		9/9	9/9	10/10	10/10	5/10	10/10	9/10	9/10	7/9	5/10		9/9	9/9	10/10	9/9	6/10	

* The numerator represents the number of mice that succumbed to the infection and the denominator the number of mice used in the test.

The *Didelphis marsupialis* tested showed no susceptibility to the virus.

Cross immunity tests in mice with opossum virus A and meningopneumonitis virus

Several groups of adult mice were immunized with the meningopneumonitis virus by the intraperitoneal inoculation of 0.2 ml of 10% mouse-brain suspension. At the same time other groups of adult mice of the same age were immunized with the caluromys and didelphis strains of opossum virus A, receiving 1.0 ml of mouse brain suspension from paralyzed mice.

After 30 days cross immunity tests were made by means of challenging intracerebral inoculation of the immunized mice (table 5). The mice immunized with the meningopneumonitis virus showed protection against the subsequent inoculation of opossum virus A, as solid as the protection against inoculation of the homologous virus (meningopneumonitis). The mice immunized with opossum virus A showed no protection against the subsequent inoculation of the meningopneumonitis virus even in the groups challenged with a small dose of virus.

Test for immunity to meningopneumonitis virus in pigeons previously inoculated with opossum virus A

Five pigeons used in the test for susceptibility to opossum virus A were challenged by intracerebral inoculation with meningopneumonitis virus (table 6). It was possible to recover virus from the blood stream of 4 of the 5 birds thus challenged. Three of these birds showed neurological symptoms and died on the 3rd, 7th and 13th day, respectively, after inoculation. Elementary bodies were demonstrated in smears prepared from the brain of each dead bird, and virus was recovered, by means

TABLE 6.—*Results of intracerebral challenge inoculation with meningopneumonitis virus in pigeons previously inoculated with opossum virus A**

Animal no.	Results of tests for circulating virus (days after inoculation)					Showing symptoms	Death or survival in days†
	3	4	5	6	7		
A.3772	0/2‡ (2/2)	0/4 (4/4)	0/4 (4/4)	0/4 (4/4)	0/3 (3/3)	No	S 60
A.3781	0/3 (2/3)	3/5 (2/2)	5/5	4/4		Yes	D 7§
A.3782	0/4 (4/4)	0/5 (5/5)	0/4 (3/4)	½/4 (3/3)	1/3 (2/2)	No	S 60
A.3783	3/4	4/5 (1/1)				Yes	D 3§
A.3784	4/4	5/5	5/5	4/5 (0/1)	5/5	Yes	D 13§
			Immune controls				
A.3666‖	0/4 (4/4)	0/4 (4/4)	0/5 (5/5)	0/5 (5/5)	0/5 (5/5)	No	S 60
			Normal controls				
A.3796	0/5 (5/5)	2/5 (2/3)	0/4 (3/4)	3/5 (2/2)	0/5 (4/4)	No	S 60
A.3797	2/4 (2/2)	4/4				Yes	D 3§

* All the birds were inoculated with the meningopneumonitis strain F.97-27A—M.150—E.3—Bogotá 57th mouse intracerebral passage, receiving 0.03 ml of 10% mouse brain suspension intracerebrally.

† S=survival; D=death.

‡ The numerator represents the number of mice that succumbed to the infection and the denominator the number of mice used in the test. The figures in parenthesis give the results of the challenging inoculation.

§ Virus was recovered from the brain and liver.

‖ Pigeon first inoculated with opossum virus A, subsequently challenged intramuscularly with meningopneumonitis virus, and virus recovered from the blood stream on the 3rd, 4th, 5th, 7th, and 8th days after inoculation.

of intracerebral inoculation into mice, from separate suspensions of liver and brain of each bird.

Neutralization test

A preliminary experiment showed that meningopneumonitis virus inoculated intraperitoneally, in an amount of 0.25 ml of 10% mouse-brain suspension into 21- to 25-day-old mice simultaneously with an intracerebral injection of starch solution, killed the mice regularly between the 4th and 7th day after inoculation. With this fact as a basis, neutralization tests were undertaken.

Dilutions of 1:20, 1:100 and 1:500 were prepared from brains of paralyzed mice infected intracerebrally with meningopneumonitis virus. Each dilution was mixed with an equal part of serum from mice immunized with meningopneumonitis virus. Another set of mixtures of each dilution and an equal part of serum from mice immunized with opossum virus A was prepared also. As control, serum from normal mice was used. This, like the other serum, was mixed with the virus dilutions. Each mixture was injected intraperitoneally into three 21-day-old mice in an amount of 0.5 ml. The mice received simultaneously 0.03 ml of 2% starch in saline solution by intracerebral inoculation. The mice were observed for 20 days, and the results were tabulated in terms of mortality and average survival time, as is usual in yellow fever protection tests[9] (table 7).

With the use of this technique it was possible to demonstrate the specific antibodies for the meningopneumonitis virus in some of the animals exposed to this virus. It showed that there were neutralizing antibodies to meningopneumonitis virus in the serums from mice and from a pigeon exposed to this virus, but that no such antibodies were

9. Bugher, J. C. 1940, The demonstration of yellow fever antibodies in animal sera by the intracerebral protection test in mice. Am. J. Trop. Med. **20**: 809–841.

TABLE 7.—*Neutralization test carried out with meningopneumonitis virus.*

Serum source	Dilution of virus in test	Mouse mortality	Average* survival time
Metachiropa A.3624 (see table 3)	1:40	3/3†	3.6
	1:200	3/3	5.0
	1:1000	1/3	15.3
D. marsupialis A.3633 (see table 3)	1:40	3/3	5.3
	1:200	3/3	6.3
	1:1000	2/3	13.0
D. paraguayensis A.3746 (see table 4)	1:40	3/3	7.0
	1:200	3/3	6.7
	1:1000	3/3	8.0
D. paraguayensis A.3747 (see table 4)	1:40	3/3	8.0
	1:200	2/3	12.7
	1:1000	0/3	20.0
Columba sp. A.3666 (see table 6)	1:40	0/3	20.0
D. paraguayensis normal serum	1:40	3/3	4.0
	1:200	3/3	5.6
Serum from mice immunized with meningopneumonitis virus	1:40	2/3	12.7
	1:200	0/3	20.0
	1:1000	0/3	20.0
Serum from normal mice	1:40	3/3	4.0
	1:200	3/3	5.0
	1:1000	2/3	11.7
Serum from mice immunized with meningopneumonitis virus	1:40	2/3	18.3
	1:200	0/3	20.0
	1:1000	0/3	20.0
Serum from mice immunized with opossum virus A	1:40	3/3	5.7
	1:200	3/3	5.7
	1:1000	0/3	20.0
Serum from normal mice	1:40	3/3	6.4
	1:200	3/3	6.4
	1:1000	2/3	12.3

* The mice were observed for a period of 20 days.
† The numerator represents the number of mice that succumbed to the infection and the denominator the number of mice used in the test.

present in the serums of any of the animals inoculated with opossum virus A, with the exception of mice, which showed some neutralizing antibodies.

DISCUSSION

The results of the study reported in this paper prove that a virus isolated from marsupials and designated as opossum virus A belongs to the lymphogranuloma-psittacosis group of viruses. Information obtained in the course of the study seems to indicate that opossum virus A is a new member of the lymphogranuloma-psittacosis group. Two strains of the virus were isolated and were shown to be identical.

In table 8 opossum virus A is compared with other members of the lymphogranuloma-psittacosis group. It is seen that this virus is pathogenic for mice when inoculated intranasally or intracerebrally but not when inoculated intraperitoneally. It is not infectious for guinea pigs when inoculated by the intraperitoneal or the intracerebral route or for pigeons when inoculated by the intracerebral or the intramuscular route, and its development is not inhibited by the sulfonamides. Thus it can be differentiated from human pneumonitis SF virus,[14] which is pathogenic for guinea pigs and pigeons; from mouse pneumonitis virus,[15,16] which is not pathogenic for mice when inoculated intracerebrally and which is inhibited by sulfonamides;[8,17,30,31] from feline pneu-

TABLE 8.—*Summary of comparison between viruses of the lymphogranuloma-psittacosis group.*

Agent	Origin	Pathogenicity for							Susceptibility to sulfonamides	References
		Mice			Guinea pigs		Pigeons			
		IN	IC	IP	IP	IC	IC	IM		
S-F virus	Human	+	+C	O NC	±	±	±C		No	14, 25, 30, 31
Mouse pneumonitis virus	Mouse	+	O	O NC	O				Yes	8, 15, 16, 17, 25, 30, 31
Feline pneumonitis virus	Cat	+	±	±					No	6, 8, 18, 30, 31
Louisiana pneumonitis virus	Human	+	+	+	+				No	19, 20, 21–24, 30, 31
Ornithosis virus	Pigeon	+	+	±C			+C	O	No	12, 13, 25, 30, 31
Psittacosis virus	Parrakeet or parrot	+	+	+C	O	±	+C		Yes	10–13, 25, 30, 31
Meningo-pneumonitis virus	Ferret	+	+	±C	±	±	+C		No	1, 10, 14, 17, 25, 30, 31
Lymphogranuloma venereum virus	Human	+	+C	O NC	O		O NC		Yes	17, 25–28, 30
Opossum virus A	Marsupial	+	+C	O C	O	O	O NC	O	No	
Opossum virus B	Marsupial	+	O	O	O	O			No	32

IN = intranasal inoculation.
IC = intracerebral inoculation.
IP = intraperitoneal inoculation.
IM = intramuscular inoculation.
+ = generally fatal.
± = occasionally fatal.
O = no effect.
C = carrier stage.
NC = no carrier stage.

monitis,[18] which is infective for mice when inoculated intraperitoneally in sufficient quantities;[6] from Louisiana pneumonitis virus, which induces fatal infection in guinea pigs inoculated intraperitoneally and kills mice by every route of inoculation;[19,24] from ornithosis and psittacosis viruses[10–13] and from meningopneumonitis virus,[1] which are all virulent for pigeons when inoculated intracerebrally and for mice when inoculated intraperitoneally. The fact that meningopneumonitis virus was shown to have a lower pathogenicity for the marsupials *Caluromys laniger* and *Metachirus nudicaudatus* than opossum virus A, as well as the results obtained in cross immunity tests with meningopneumonitis virus and opossum virus A (tables 5 and 6), provides additional evidence that these two agents are different viruses. The protection against a challenging inoculation with opossum virus A demonstrated in the mice immunized with meningopneumonitis virus is in accordance with the previous demonstration by Beck et al[25] of the broad antigenic action of the meningopneumonitis virus for other members of the lymphogranuloma-psittacosis group (ornithosis, psittacosis and human pneumonitis SF viruses).

The opossum virus A has more properties in common with the lymphogranuloma venereum virus than with

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any other member of the lymphogranuloma-psittacosis group; i.e., intranasal and intracerebral pathogenicity for white mice, no virulence for these animals when the virus is given intraperitoneally and no pathogenicity for pigeons and guinea pigs. But the two viruses can be differentiated by certain characteristics: (a) Jones et al[26] have recorded the persistence of the virus of lymphogranuloma venereum in the brains of mice 3½ months after intracerebral inoculation of the virus, whereas the carrier stage for the opossum virus A in white mice was demonstrated only up to 21 days after inoculation of the virus. (b) The susceptibility of the lymphogranuloma venereum virus to the action of sulfonamides has been demonstrated by Findlay,[27] Jones et al,[26] Rake et al,[17] van den Ende and Lush,[28] Jones et al[29] and Meiklejohn et al,[30] while it may be concluded from the results obtained in this study that the sulfonamides do not have any inhibiting effect on the opossum virus A.

SUMMARY

1. A meningopneumonitis virus which forms elementary bodies was isolated from the Colombian opossums *Didelphis paraguayensis* and *Caluromys laniger*. The virus was provisionally named "opossum virus A."

2. The virus was pathogenic for opossums (with the exception of *Didelphis marsupialis*) when it was injected intraperitoneally; it was mainly so for *Metachirus nudicaudatus* and *Caluromys laniger*. From the experimentally infected opossums the virus was recovered from the blood stream from the 3rd to the 9th day after inoculation, and it was recovered also from the organs and peritoneal exudate of those animals which died.

3. The virus was fatal to white mice when it was inoculated by the intracerebral or intranasal route, producing a meningitis or pneumonitis according to the route of infection. It could be maintained in serial passages in white mice when inoculated by either the intracerebral or intranasal route. It was not infectious for white mice when injected intraperitoneally even in large amounts, but the mice injected by this route showed subsequent resistance to infection on intracerebral inoculation with the same virus.

4. The virus was not pathogenic for guinea pigs when injected in large amounts intraperitoneally or intracerebrally. It did not show virulence for pigeons inoculated either intramuscularly or intracerebrally, but in one instance a trace of virus was recovered from the blood stream of 1 of 9 pigeons

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inoculated. None of the inoculated pigeons showed symptoms.

5. The virus was cultured in the yolk sac of chick embryos, with the production of an endotoxin which killed mice when it was inoculated intravenously. The virus was maintained by serial passages in the chick embryos.

6. The virus was cultivated in serial passages in tissue cultures prepared with mouse and chick embryos.

7. The experimental infection induced in mice inoculated intracerebrally or intranasally was not inhibited by sulfonamides.

8. Laboratory data have been presented which show that opossum virus A belongs to the lymphogranuloma-psittacosis group and that it can be differentiated from the other members of that group.

THE SEROLOGICAL CLASSIFICATION OF THE BALLERUP GROUP OF PARACOLON BACTERIA

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The Ballerup group of paracolon bacteria is composed of microorganisms related directly or indirectly to the culture described by Kauffmann and Moeller[1] as *Salmonella ballerup*. They may be defined as motile or nonmotile coliform bacteria which produce abundant hydrogen sulfide but fail to form indol; usually are methyl red-positive; are Voges-Proskauer negative; promptly produce acid and gas from glucose, arabinose, maltose, rhamnose, xylose, trehalose, mannitol, or sorbitol; attack lactose, sucrose, dulcitol, or salicin with varying avidity but fail to ferment inositol; utilize d-tartrate; usually utilize sodium citrate; and rarely liquefy gelatin. Of 47 cultures classified, one liquefied gelatin. Two strains did not ferment salicin upon prolonged incubation, 13 strains did not attack dulcitol, 3 strains did not utilize sodium citrate, and 9 strains were methyl red-negative. Nine strains could not be induced to attack lactose or sucrose although the majority of the 47 strains fermented one or both of these sugars. The speed of fermentation varied from the production of acid and gas within 24 hours to the appearance of slight acidity after serial transfers over periods of 1 to 6 months in the presence of lactose or sucrose in sealed fermentation tubes.

The methods used in the serological study of the organisms were those outlined by Edwards and Bruner,[2] and Edwards, West, and Bruner.[3] The sources of the cultures studied were: man (40), a hog (1), a hen (1), a duck (1), oysters (1), sewage (1), unknown (1), cream puff (1).

Somatic antigens

S. ballerup was assigned the somatic formula XXIX [Vi] by Kauffmann and Moeller.[1] The culture possessed the V-W variation described by Kauffmann.[4] Of the 47 cultures studied, Vi antigen was detected in 21. This was done by plating and examining single colonies. V colonies of the Ballerup group appear more opaque to the naked eye than do W colonies, and they agglutinate readily on a slide when exposed to pure Vi antiserum.

Somatic relationships were established by using each culture in agglutinin-absorption tests. Agglutination tests with alcohol-treated cultures indicated that the 47 cultures were divisible into 3 major somatic groups. In table 1 are shown the agglutination reactions of these somatic groups and subgroups. One strain of each O type and their respective serums are in-

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3. Edwards, P. R., West, Mary G. and Bruner, D. W. 1947, Ky. Agr. Exp. Sta. Bull. 499.
4. Kauffman, F. 1936, Ztschr. f. Hyg. u Infektionskr. **117**: 778.

cluded. Table 1 also lists the Arabic numerals and Roman letters assigned to the O factors of the various somatic groups and subgroups. Somatic group 1 was divisible into 5 subgroups readily distinguished by absorption tests. The common antigen possessed by the members of these subgroups was designated by the symbol 1a, and the subgroups were indicated by the symbols 1a, 1b; 1a, 1b, 1c; 1a, 1b, 1d; 1a, 1d, 1e; and (1a), 1f. The parentheses signify that part of antigen 1a is lacking. The specific serums for diagnostic use were prepared by absorption, i.e., single factor 1c serum was obtained by absorption of 1a, 1b, 1c serum by 1a, 1b antigen. The remaining 2 somatic groups were designated by figures 2 and 3, respectively. Although it readily was possible to distinguish the 3 somatic groups by slide agglutination, it was necessary in most cases to use tube tests to determine the 5 subgroups of somatic group 1. Specific factor 1c could be demonstrated by slide agglutination. Apparently the other specific factors were too weak to be manifested in this less sensitive test. A detailed study of the O antigens of the Ballerup group has been published by Bruner et al.[5]

TABLE 1.—*Agglutination reactions of somatic groups.*

Antigens		Antiserums						
		LC54	H8	527	4924	3153F	3796	5818
Cultures	O Factors	1a, 1b	1a, 1b, 1c	1a, 1b, 1d	1a, 1d, 1e	(1a), 1f	2	3
LC54	1a, 1b	2000	2000	2000	500	1000	—	—
H8	1a, 1b, 1c	2000	2000	1000	500	1000	—	—
527	1a, 1b, 1d	2000	2000	2000	1000	200	—	—
4924	1a, 1d, 1e	1000	1000	2030	2000	500	—	—
3153F	(1a), 1f	1000	1000	1000	200	2000	—	—
3796	2	—	—	—	—	—	2000	—
5818	3	—	—	—	—	—	—	2000

Figures indicate highest dilution at which agglutination occurred.
— indicates no agglutination at 1:100.
Parentheses indicate part of antigen lacking.

Flagellar antigens

Nine of the 47 cultures proved to be nonmotile. In the agglutination tests for H factors, formalinized broth cultures of actively motile bacilli were used as antigens. The tubes were incubated in a water bath at 48 C and read after one hour.

The Arabic numerals assigned to the H factors of the Ballerup group are given in table 2. The serum strains and the absorbing strains used to determine the single factors also are given. It was noted that culture 5818 was agglutinated by serums possessing factor 3 but did not remove all of the fraction from 3 serum. The possession of part of antigen 3 was signified by parentheses. Likewise, cultures H10 and 20964 lack part of antigen 5. The H factors given in table 2 are those of diagnostic importance and do not in all cases represent the complete antigenic pattern of the cultures.

It is indicated in table 2 that the Arabic number 1 represents the *Salmonella* symbol z_{14} which was assigned to the H antigens of *S. ballerup* (Kauffmann and Moeller[1]). Also, numbers 2, 3 represent the *Salmonella* symbols assigned to the H antigens of *Salmonella hormaechei* (XXIX, [Vi]: z_{30}, z_{31}) of Monteverde (Edwards[6]). Figure 2 signi-

5. Bruner, D. W., Edwards, P. R. and Hopson, Alice S. Ky. Agr. Exp. Sta. Bull. In press.

6. Edwards, P. R. 1946, J. Bact. **51**: 523.

TABLE 2.—*Ballerup diagnostic serums (H factors).*

Ballerup factors	Antiserum	Symbols of components		Absorbing strains and Ballerup components
		Ballerup	*Salmonella*	
1	107	1	z_{14}	Unabsorbed
2	LC54	2, 3	z_{28}, z_{31}	Horm-3
3	LC54	2, 3	z_{28}, z_{31}	N153-2
4	5818	(3), 4		Horm-3
5 . . .	8665	5, 6		Unabsorbed
6	8665	5, 6		527-5, 7
7	527	5, 7		8665-5, 6
8	H10	(5), 8		8665-5, 6
9	20964	(5), 9		8665-5, 6
10	16385	10		Unabsorbed
11	Dyer	11		Unabsorbed
12	H8	12		Unabsorbed
13	59	13		Unabsorbed
14	14671	14		Unabsorbed
15	17776	15		Unabsorbed
16	2058	16		Unabsorbed
17	48203	17 . . .		LC54-2, 3

. . . indicates abbreviated formula.
Parentheses indicate part of antigen lacking.

fied symbol z_{30}, and figure 3 takes the place of symbol z_{31}.

Classification

After establishing the O and H antigens of the Ballerup group, it was necessary only to correlate the results of the examination of the O and H antigens of each of the 47 cultures studied in order to determine the serological types. This is done in table 3. When all the cultures which possessed O antigens 1a, 1b were brought together, it was found that their H antigens fell into 5 groups represented respectively by H factors 2; 2, 3; 15; 16; and 17. Thus 5 serological types as well as a group of nonmotile cultures are present in O subgroup 1a, 1b. The presence of Vi antigen is signified by adding symbols Vi to the somatic formulas, and its presence in the serological types delineated is indicated in table 3.

In writing the formulas of these strains the letters "Bal." are used to denote the Ballerup group. The formula for the first type given in table 3 is Bal. O-1a, 1b, 1c, [Vi]: H-1, or, simply, Bal. 1a, 1b, 1c, [VI]: 1. Culture H8 which lacks Vi antigen would be Bal., 1a, 1b, 1c: 12. It was found that the three O groups were divisible into 21 types on the basis of their H antigens.

Table 3 also lists the cultures representing the serological types. In many cases they were represented by a single culture. As larger numbers of cultures are examined, the number of types will increase through new antigenic combinations and the recognition of additional O and H antigens.

Pathogenicity

Little is known about the pathogenicity of this group of paracolon bacteria. The original Ballerup strain was isolated from the feces of a woman who was suffering from gastroenteritis. At least 13 of the 47 cultures typed were associated with gastroenteritis in man. One culture came from sewage, one from the feces of a normal hog, one from the feces of a duck, one from the ovary of a hen, one from oysters, and one from a cream puff. A number of the cultures were derived from asymptomatic human carriers. Since these microorganisms were isolated in some instances from cases of diarrhea in man in which no other inciting bacteria were found, it is possible that they were the causative agents. Animals apparently carry paracolon bacteria of this group and may be important in spreading them.

DISCUSSION

The original Ballerup strain was placed in the *Salmonella* group when it was described by Kauffmann and Moeller.[1] The culture possessed Vi antigen, an antigen shared by *Salmonella typhi* and *Salmonella paratyphi C*. Neither its O antigens, XXIX, nor its H antigens, z_{14}, were found in other members of the *Salmonella* group. Later, it was found by Kauffmann[7] that Vi antigens occurred in coliform strains, so

TABLE 3.—*Proposed diagnostic schema for bacteria belonging to the Ballerup group.*

Group	O	H	Cultures classified
1	1a, 1b, 1c	1*	107, Fels, H1, Lille
		2*	8382, 223, 294, 17016, 695, 49221, 49223
		5, 6*	8665, 3205, 7–39, H2, H6, L81b
		5, 7*	79
		(5), 8	H10
		(5), 9	20964
		10*	16385
		11*	Dyer
		12	H8
	1a, 1b, 1d	3*	Horm
		5, 7	527, 5096
		13	59
		14	14671
	1a, 1b	2*	M, N153
		2, 3*	LC54
		15*	17776
		16*	2058
		17	48203, 48204
			6699, 1938, 1939, H3, H4, H5, H7, H9
	1a, 1d, 1e		4924
	(1a), 1f	(2), 3	3153F
2	2	1	3796
3	3	(3), 4	5818

Parentheses indicate part of antigen lacking.
The presence of Vi antigen is indicated in the O formulas of members of the Ballerup group in the usual manner, as 1a, 1b, 1c [Vi]: 1 for cultures 107, Fels, H1 or Lille.
* Vi antigen detected in at least one member of these types.

that the presence of this component can no longer be regarded as grounds for including the Ballerup strain in the genus *Salmonella*. The microorganism conforms to the biochemical characteristics of the genus *Salmonella* when examined by the usual methods. However, it is a slow salicin fermenter and can be forced to ferment lactose with acid and gas production when cultured serially in the presence of this sugar in sealed fermentation tubes. Since members of the *Salmonella* group ordinarily are not rapid salicin fermenters nor do they ferment lactose, the Ballerup strain does not conform entirely to the genus definition of *Salmonella*. The fact that it shares no antigens other than Vi with *Salmonella* types adds weight to the argument for including it in a paracolon group.

Harhoff[8] examined 24 strains of Ballerup bacteria. His strains were included in this study along with 23 additional cultures. He established three O types and designated them $XXIX_1$, $XXIX_2$, $XXIX_3$; $XXIX_1$, $XXIX_2$; and $XXIX_1$, $XXIX_3$. We assigned symbols 1a, 1b, 1c to the members of his $XXIX_1$, $XXIX_2$, $XXIX_3$, O type and symbols 1a, 1b to the members of his $XXIX_1$, $XXIX_2$ O type. Harhoff stated that the results he obtained in studying the members of his $XXIX_1$, $XXIX_3$, O type varied, and our results indicated the necessity of splitting this subgroup into the three O types 1a, 1b, 1c; 1a, 1b, 1d; and 1a, 1b, 1e. Classification of additional cultures not possessed by Harhoff led to the delineation of a fifth somatic subgroup (1a), 1f, within group 1, and to the setting up of somatic groups 2 and 3.

In establishing the H antigenic relationships we were essentially in agreement with the results obtained by Harhoff in studying 16 motile Ballerup strains. However, table 3 shows a much more complete antigenic analysis of the

7. Kauffmann, F. 1941, Die Bakteriologie der Salmonellagruppe. Kopenhagen, Einar Munksgaard.
8. Harhoff, N. 1949, Acta. path. et microbiol. Scandinav. **26**: 167.

H antigens of the Ballerup paracolon group than he set forth.

Observed phase variation in the Ballerup group was limited to a form of dissociation into loss variants (Edwards[5]) and induced transformation of H antigen 1 to H antigen 3. Culture LC54 (Bal. 1a, 1b: 2, 3) possessed flagellar antigens in a 2, 3 form, a 2 form, and a 3 form. Starting with a single colony Bal. 1a, 1b: 2, 3 it readily was possible to induce a Bal. 1a, 1b: 2 phase or a Bal. 1a, 1b: 3 phase. No evidence of diphasic Ballerup microorganisms as seen in many types of the genus *Salmonella* was noted. An attempt was made to split culture 8665 (Bal. 1a, 1b, 1c: 5, 6) into H phases 5 and 6 and to change H phase 5, 6 to 5, 7. These changes were not accomplished.

SUMMARY

A group of 47 cultures of Ballerup paracolon bacteria was examined. The group was defined and antigenic formulas were assigned to the various types. The 47 cultures were divisible into three O groups and 21 serological types. They were isolated from man, a hog, a hen, a duck, oysters, sewage, and a cream puff. Although many of the cultures were derived from asymptomatic carriers, at least 13 of the 47 were associated with gastroenteritis in man. Nineteen cultures possessed Vi antigen and three of these cultures were identical with the original Ballerup strain. All cultures were monophasic in character, but a form of loss variation in the H antigens was encountered.

STUDIES WITH *SHIGELLA DYSENTERIAE* (SHIGA). III. INFECTION AND INTOXICATION IN *MACACUS MULATTA* MONKEYS

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Infections with *Shigella paradysenteriae* (Flexner) occur spontaneously in monkeys, and epidemics within a colony are not uncommon. Infections with *Shigella dysenteriae* (Shiga) have not been described in these animals.

Whether or not it is possible to produce *S. dysenteriae* infection in monkeys is a question of more than academic interest. The study of the clinical and pathological picture produced in monkeys by *S. dysenteriae* (Shiga) and its toxins might give useful information about this relatively rare type of dysentery and afford an opportunity to study its treatment and prevention as well. The possibility of producing infection or intoxication of monkeys with *S. dysenteriae* (Shiga) or toxins prepared from this organism was investigated in this study, and the results are herein reported.

STUDIES WITH SHIGA CULTURES IN MONKEYS

All of the monkeys used were *Macacus mulatta*, and they varied considerably in size and age.

Twenty-two strains of *S. dysenteriae* (Shiga) were used in these studies. None of these were freshly isolated, though 6 were obtained during the recent war—2 from North Africa and 1 from Syria were received through Dr. L. A. Barnes of the Naval Medical Center, and 3 from Cairo were sent by Dr. J. S. K. Boyd. The remainder had been isolated in the United States previously—4 from an epidemic in Michigan, 2 from an outbreak in Adair County, Kentucky, and 10 from sporadic cases in Mississippi. All of these had been kept "smooth" by frequent platings, and most of them showed considerable virulence for mice when injected intraperitoneally together with mucin. They were routinely preserved in a dried state but were transferred twice a day for several days before use. Five-hour broth cultures were generally used in experiments in which single strains were employed. Such cultures usually contained about 1 billion organisms per ml. When a heavier suspension or a pool of strains was desired, the growth from 5-hour agar slant cultures was suspended in broth and adjusted to the desired density.

Among those monkeys given cultures of *S. dysenteriae* by mouth, rectal temperatures and stool samples were taken daily. The fecal specimens were taken on ordinary cotton-tipped wooden applicators and each placed in a test tube for transportation to the culture room. They were plated out as soon as possible on "SS" agar, and colonies resembling those of *Shigella* were picked after 24 and 48 hours incubation to Russell's or Kligler's agar slants. From these identification was completed by fermentation and agglutination reactions.

Cultures given by mouth.—The animals were anesthetized by nembutal, given intravenously. Cultures were administered by stomach tube. Three groups of 4 monkeys each were fed 5-hour broth cultures of mouse-virulent Shiga strains. The first group was given 2 billion bacteria in a volume of 5 ml; 2 monkeys (no. 1 and 2) received strain D6 (Mississippi) and 2 (no. 3 and 4) D21 (North Africa). The monkeys showed

no apparent discomfort, and there was no significant rise in temperature at any time. *S. dysenteriae* (Shiga) was recovered from the stools of 3 of these 4 monkeys taken 24 hours after the culture was administered but not from subsequent samples.

The second group of 4 monkeys received 8 billion bacteria; no. 5 and 6 received strain D10 (Mississippi) and no. 7 and 8, D2O (Kentucky). This dose of organisms contained in 8 ml broth was washed down with 10 ml of 10% sodium bicarbonate to neutralize the effect of gastric acidity, and the animals were given $\frac{1}{4}$ grain of morphine intraperitoneally to retard peristalsis. There were no apparent symptoms and no significant changes in temperature among the animals in this group. *S. dysenteriae* (Shiga) was recovered at 24 hours from 2 of the monkeys, but not later.

These first 2 groups of monkeys had received single strains of Shiga bacilli chosen because of their marked virulence for mice. Following the report of Shaughnessey and his coworkers[1] on the lack of correlation between virulence for mice and that for man among single Flexner strains, the next 4 monkeys (no. 9, 10, 11, and 12) were fed with pools A or B, each containing 10 strains, the dose given totalling 100 billion Shiga cells per monkey. These heavy suspensions were prepared by washing the growth from 5-hour agar slants and adjusting this suspension to the desired density by means of turbidity standards. This dose (in 10 ml) was also followed by 10 ml of 10% sodium bicarbonate. No morphine was given. With this group also there were no symptoms nor significant change in temperature. *S. dysenteriae* (Shiga) was recovered from stool samples taken 24 hours after feeding from all 4 monkeys and from 2 of them taken at 48 hours.

Subsequently, two monkeys (no. 13 and 14) were each fed pool C, consisting of a suspension of 100 billion Shiga cells, representing 11 different strains, in 5 ml of 5% mucin. Neither sodium bicarbonate nor morphine was given to the monkeys, but the suspension was washed down with a few ml of physiological salt solution. No symptoms followed and no significant increase in temperature occurred. The stools of both animals yielded *S. dysenteriae* (Shiga) the day after ingestion, and this organism was also recovered from monkey no. 14 seven days later.

Cultures given parenterally.—A total of 25 monkeys were injected intraperitoneally with 5-hour agar growth of Shiga culture in 5 ml of 5% mucin. The dose given to 13 (monkeys 15 to 27 inclusive) contained 5 million Shiga cells of single mouse-virulent strains. Ten of these monkeys seemed unaffected, but on the second day 2 (monkeys 25 and 26) developed profound weakness and pronounced tremor of all skeletal muscles, which increased when they were handled. After the third day, monkey 25 began to improve and eventually recovered. Monkey 26 grew progressively worse, developed a meningitis, and died on the 16th day. Cultures from brain, spinal fluid, and blood were negative. A third monkey (no. 20) developed a paralysis of the lower extremities on the 6th day after injection and was found dead on the 7th day. Autopsy revealed a purulent abscess at the site of the injection. From this abscess and from the heart blood *S. dysenteriae* (Shiga) was recovered. This was the only monkey of this group

1. Shaughnessy, H. J., Olson, R. C., Bass, K., Friewer, F. and Levinson, S. O. 1946, Experimental human bacillary dysentery; polyvalent dysentery vaccine in its prevention. J.A.M.A. **132:** 362–368.

from which a positive blood culture was obtained.

In a like manner, 4 monkeys (no. 28 to 31 inclusive) were given 10 million Shiga cells of other single mouse-virulent strains. These animals were obviously uncomfortable for about an hour after injection. All showed some degree of prostration during that time, and one vomited and assumed a knee-chest position. All seemed normal by the next morning.

The remaining 8 monkeys of this series were, in the same manner, injected with pools of 10 strains of *S. dysenteriae* in doses of 100 million, 1 billion, and 20 billion organisms. The two given 100 million (monkeys 32 and 33) showed mild malaise and loss of appetite for about 2 days. The animals were able to climb about the cage when disturbed. By the 4th day they seemed entirely normal. The two given 1 billion Shiga bacilli (monkeys 34 and 35) were more definitely ill, and by the 3rd day were very weak, moving about with difficulty. Their abdomens were distended. Blood and peritoneal fluid were withdrawn for culture from both of these monkeys. *S. dysenteriae* (Shiga) was recovered from the peritoneal fluid of one monkey but not from the blood of either animal. Both of these monkeys survived. On the other hand, all 4 monkeys receiving 20 billion Shiga bacilli intraperitoneally died. These animals seemed very uncomfortable immediately after injection and showed a marked pallor; within an hour they were prostrate or else sat with head bowed down over the feet. Two died within 24 hours (monkeys 36 and 37), one in 41 (monkey 39), and the 4th (monkey 38) in 70 hours. This last monkey (no. 38) developed a spastic paralysis of the legs on the 2nd day and shuffled about with difficulty when disturbed.

At autopsy these last 4 monkeys all showed petechiae on the skin of the axillary and inguinal regions. There was an acute hemorrhagic peritonitis; the omentum and the intestinal tract were glued together in a hemorrhagic fibrinous mass. There was considerable bloody peritoneal fluid, and in monkey 39 there was bloody pleural fluid also. Cultures were made from peritoneal, pleural, and spinal fluid, and from heart blood, as well as from several organs. Shiga bacilli were recovered from blood, peritoneal fluid, and spleen of 3 of these 4 monkeys. However, it was not recovered from any organ or tissue of monkey 38, the one that lived 70 hours after injection, although the autopsy was done immediately after death. This circumstance suggested that death might have been due to the toxins of the Shiga bacilli rather than to infection.

Histological studies of the 6 monkeys that died following intraperitoneal injection of Shiga cultures in mucin were made. The general picture was that of an acute peritonitis. There were, however, other findings characteristic of toxin action. These will be discussed later in the section on pathologic histology.

STUDIES WITH SHIGA TOXIN IN MONKEYS

Oral administration of toxin.—Eight monkeys were fed large doses of unaltered Shiga toxins. These preparations, made by the method of Farrell and Ferguson,[2] contained both neuro- and enterotoxin.

The toxins were administered to the monkeys by means of a stomach tube, employing nembutal anesthesia, in the same manner in which the living organisms had been given. The toxin was preceded by 10 ml of 10% sodium bicarbonate to neutralize gastric acidity,

2. Farrell, L. and Ferguson, H. 1943, Shiga toxoid. Canad. J. Pub. Health, **34**: 130–139.

and with the first 4 monkeys ¼ grain morphine was injected to retard peristalsis.

The first 2 monkeys of this series (no. 40 and 41) were given 5 ml of "pork toxin" #1, diluted 1:5. This was made from Shiga strain 7585. Since 1 ml of the 1:200 dilution of this toxin was subsequently found to be fatal for monkeys on intravenous injection, the amount of toxin fed was equivalent to 200 fatal i.v. (intravenous) doses. Considerable difficulty was encountered in passing the tube into the stomach of monkey 41, and it was believed that some of the toxin went into the trachea. Except for seeming somewhat "slowed" in his reactivity for a couple of days, monkey 40 showed no effects from the toxin. Monkey 41 developed quick and labored respiration on the second day and became somewhat lethargic. By the third day he was very irritable and had generalized tremors. A definite nystagmus and a marked weakness of the legs followed. The weakness increased, and the animal died on the 5th day. This monkey developed a hemorrhagic rash, numerous petechiae in axillary and inguinal regions, and large ecchymotic patches on the surface of the abdomen. Autopsy revealed a few petechial hemorrhages in the omentum and in the mesenteric and inguinal lymph nodes. There was also hemorrhage in the walls of the large intestine. There was patchy consolidation of the lungs, and adhesion of the lungs to the chest wall. The trachea was filled with thick mucus. The heart wall was very purple and seemed to be solidly hemorrhagic.

The next 2 monkeys (no. 42 and 43) were given 1000 fatal i.v. doses of the same toxin by mouth, together with the sodium bicarbonate and morphine. These animals were rather listless for several days. Monkey 42 showed muscle weakness when tired, and no. 43 developed a definite weakness of the left leg, although he could use it when forced to do so. Both of these animals recovered within a week.

Two other monkeys (no. 44 and 45) were given 5 ml of undiluted "beef toxin" #1, also prepared with strain 7585. Since 1 ml of the 1:400 dilution of this toxin was fatal to monkeys when injected intravenously, these animals were actually fed 2000 fatal i.v. doses. The sodium bicarbonate was administered just before the toxin, as with the preceding monkeys, but the morphine was omitted. These 2 monkeys showed no symptoms whatever aside from an apparently increased excitability on the following day. In addition to these 8 monkeys that were given toxin by mouth, 2 others (no. 46 and 47) were fed 10 ml of the undiluted toxin in which were suspended approximately 60 billion Shiga bacilli. No sodium bicarbonate or morphine was given. No symptoms of any kind were noted in these monkeys, but Shiga organisms were recovered from the stools of both animals on the day after feeding and from one (no 47) on 5 consecutive days thereafter.

Two monkeys (no. 48 and 49) were given undiluted toxin BR1 (from strain Bucharest R) intranasally under light ether anesthesia. The toxin was instilled by pipette, 1 ml into each nostril. Both animals seemed unaffected.

Experiments were done to ascertain the effect of hydrochloric acid, pepsin, and trypsin upon the toxin. A small amount of toxin BR1 (mouse titer = 1:200) was diluted 1:5. The pH, originally 8.2, was brought to 2.0 with $N/1$ hydrochloric acid. The toxin was now divided into two portions. One was allowed to remain in this stage; to the other 5 mg of crystalline pepsin (Difco, titer 1:10,000) was added to each 10 ml of diluted toxin. Both portions were incubated in a water bath at 37 C for

3 hours, after which they were brought to pH 7.4 with $N/1$ sodium hydroxide. To a similar sample of the original toxin, diluted 1:5, 5 mg of powdered trypsin (Difco, titer 1:250) was added to each 10 ml. The pH was allowed to remain at 8.2. A similar amount of trypsin in physiological salt solution was included as a control and both samples incubated in a water bath at 37 C for 6 hours.

All of these preparations, as well as a sample of the diluted untreated toxin, were injected into mice intravenously in dilutions of 1:5, 1:10, 1:20, and 1:40. The dose was 0.5 ml, and 4 mice were used for each dilution. These experiments were done twice with identical results.

The toxin was unaltered by trypsin. All of the mice receiving the trypsin-toxin mixture as well as all receiving the toxin only died with the typical toxin syndrome. Those receiving trypsin alone were unaffected. On the other hand, the toxin was completely destroyed by the pepsin-hydrochloric acid combination, no mice showing any symptoms. The hydrochloric acid alone affected the toxin to a considerable extent, reducing the titer by one-half.

Toxin given parenterally.—A very different chain of events followed parenteral administration of the bacteria-free Shiga toxin. In all, 34 monkeys were injected either subcutaneously or intravenously with various dilutions of toxin, although the symptoms were the same by either route. The incubation time, the degree of involvement, and the outcome were more constant when the toxin was given intravenously. Monkeys proved very susceptible, the fatal dose by the intravenous route being approximately the same as that for mice. They were given 1 ml doses of dilutions ranging from 1:5 to 1:500 in 0.85% saline. With most lots of toxin the minimum fatal dose was found to be in the 1:200 to 1:500 range.

Although the time of appearance of the first symptoms depended to some extent upon the amount of toxin given, there was a minimum incubation time which was not decreased, even by administration of undiluted toxin. The shortest intervals noted before appearance of any symptoms were 24 and 36 hours in monkeys given toxin diluted only 1:5 (no. 50 and 51 in table 1). The usual incubation time was 48 hours in instances which resulted in death, though sometimes death followed an incubation time of 4 to 5 days (no. 62 and 64). Some animals developed typical symptoms after periods as long as 6 to 10 days. In such cases complete recovery was the rule (no. 61, 63, 65, 66, and 67 of table 1). Five monkeys showed no symptoms (no. 74, 75, 77, 78, and 79).

As a rule, the severity of the intoxication was in proportion to the amount of toxin administered. There was, nevertheless, an individual difference among the monkeys. The first symptom was weakness of the legs. This was commonly followed by epileptic convulsions if the animal was handled or exercised. The monkey would fall with a type of seizure resembling that of epilepsy, with a phase of tetanic spasm followed by a phase in which the whole body was involved in clonic convulsions. This was followed by complete prostration during which the animal was limp and apparently unconscious. After a few minutes the animal was seemingly as well as before the seizure. With some monkeys the picture did not progress beyond this stage, and complete recovery ensued. With others, a typical spastic paralysis developed, beginning in the lower extremities and ascending. Prostration ensued and then death. Sometimes the whole cycle occurred so rapidly that

TABLE 1.—*Effect of intravenous injection with Shiga toxin on monkeys.*

No. of monkey	Dose (1 ml) dilution	Incubation time	Clinical picture	Outcome	Gross findings at autopsy
50	1:5	24 hr.	Prostration. Dyspnea. Spastic paralysis legs.	Death 42 hr.	Patchy congestion of lungs. Yellow pleural fluid.
51	1:5	36 hr.	Prostration. Spastic paralysis legs.	Death 44 hr.	No gross changes noted.
52	1:25	48 hr.	Prostration. Spastic paralysis both arms and legs.	Death 3 days	Hemorrhagic petechiae on skin. Hemorrhage in heart and lymph nodes.
53	1:25	48 hr.	Prostration.	Death 3 days	Punctate hemorrhage over omentum, mesenteric glands, heart.
54	1:50	48 hr.	Weakness. Spastic.	Death 3 days	Punctate hemorrhages in omentum, lung, heart.
55	1:50	48 hr.	Spastic paralysis. Tetanic convulsions.	Death 3 days	Hemorrhage into heart muscle. Punctate hemorrhage in omentum.
56	1:100	48 hr.	Spastic paralysis. Prostration.	Death 4 days	Petechiae in axillae and on eyelids. Hemorrhage in heart muscles and mesenteric glands.
57	1:100	48 hr.	Spastic when disturbed. Prostration.	Death 4 days	Petechiae in skin. Hemorrhage in lymph nodes and heart.
58	1:150	72 hr.	Tetanic convulsions. Spastic paralysis.	Death 4 days	Petechiae in skin. Hemorrhage in omentum, large intestine, heart.
59	1:200	48 hr.	Prostrate, limp, moribund.	Death 4 days	Petechial hemorrhages on peritoneal wall and omentum.
60	1:200	48 hr.	Tetanic convulsions when disturbed. Spastic paralysis.	Death 5 days	Petechiae in skin. Hemorrhage in heart muscle.
61	1:200	6 days	Slight but definite paralysis of legs.	Recovery	
62	1:200	3 days	Prostration.	Death 4 days	Regional lymph nodes congested. No hemorrhage in heart.
63	1:200	6 days	Weak and easily exhausted. No spasticity.	Recovery	
64	1:200	5 days	Weak. Prostrated. Spastic.	Death 9 days	Petechiae on skin. Hemorrhage in heart muscle and mesenteric lymph nodes.
65	1:200	10 days	Spastic paralysis of legs and partly of arms.	Recovery	
66	1:400	7 days	Very weak. Collapsed when exercised.	Recovery	
67	1:400	5 days	Tetanic convulsions when disturbed.	Recovery	
68	1:400	48 hr.	Prostration.	Death 3 days	Hemorrhage in axillary lymph nodes and large intestine. No petechiae on skin.
69	1:400	48 hr.	Spastic paralysis of whole body. Tremor.	Death 10 days	Petechiae on skin. Hemorrhage in heart, mesentery, intestine, lymph nodes.
70	1:500	48 hr.	Spastic paralysis. Tetanic convulsions.	Recovery	
71	1:500	48 hr.	Weak and easily exhausted.	Recovery	
76	1:300	48 hr.	Weak and easily exhausted.	Recovery	
80	1:200	48 hr.	Tetanic convulsions. Spastic paralysis of legs.	Death 4 days	Petechiae in axillary and inguinal skin. Hemorrhagic spots on large intestine, omentum, parietal peritoneum.
81	1:150	72 hr.	Convulsions.	Death 4 days	Hemorrhage in heart muscle. Petechiae on skin, lymph nodes, peritoneum, omentum, kidney, pancreas.
82	1:200	48 hr.	Weakness of legs. Easily exhausted.	Death 5 days	Petechiae on skin, large intestine, heart.
83	1:200	48 hr.	Convulsions (tetanic). Prostration.	Death 4 days	Petechiae on skin, hemorrhage in lymph nodes, omentum, mesentery, intestine walls, caecum, lung. Massive heart hemorrhage. Pericardial fluid.

complete prostration seemed to be the only stage recognized. Table 1 summarizes the effects of Shiga toxin given intravenously to 27 monkeys.

PATHOLOGIC HISTOLOGY

Partial post-mortem material was collected from 22 monkeys which died at varying intervals after administration of toxin (16) or intraperitoneal infection with organisms (6). In some instances post-mortem changes were rather advanced, and in these only a few organs were studied histologically.

In general the lesions encountered fell into two groups: (1) focal and diffuse hemorrhage occurring in skin, serous membranes, heart muscle, adrenal cortex, and lymph nodes and (2) parenchymatous and fatty degenerations of heart muscle, liver, and kidneys. These lesions presented little evidence of evolution by time interval. It would seem that the observed extent was that which was sufficient to cause a fatal outcome, regardless of the time interval.

Adrenal cortical hemorrhages occurred in 8 of 11 monkeys treated with toxin. They were most extensive in the 2 which died at 42 and 44 hours and were infrequent in those dying on the 5th, 8th, and 10th days. The medulla usually presented no significant lesions. Cortical lipoids were generally present in moderate amounts.

Focal hemorrhage of the adrenal cortex was noted only in 1 of 6 monkeys given *S. dysenteriae* intraperitoneally; this one died on the second day.

In 7 of 9 monkeys receiving toxin the renal convoluted tubules presented more or less numerous basally located fine fat droplets in their epithelium. This was quite variable in extent, both in number of tubules involved and in their predominant location, whether in the deep or subcapsular zones of the cortex. Usually, even in the absence of fatty changes, the epithelial cytoplasm was swollen, granular, and often more or less increasedly oxyphil. Frank coagulation necrosis of more or less extensive areas or of isolated cells was usually present, and in the later animals oxyphil granular or hemoglobin casts were seen. Intertubular or glomerular capillary thrombosis was noted in three instances, in one extending to hemorrhagic necrosis of glomeruli. Otherwise, fatty changes were noted in glomerular epithelium and in loop tubule epithelium in one monkey each.

In the 6 monkeys infected with living organisms, parenchymatous and fatty degeneration of convoluted tubules was present, but necrosis, thrombosis, and hemoglobin casts were not observed.

Minor to extensive fatty degeneration of heart muscle fibers was present in all 16 monkeys given toxin and in the 5 infected ones which died on the 1st, 1st, 1st, 3rd, and 16th days. Fat droplets were quite fine and located in large numbers throughout involved muscle fibers. Fatty changes were often more pronounced in small areas, but sometimes were diffuse, and were sometimes exceptionally severe in areas of interstitial hemorrhage. Focal or diffuse epicardial, endocardial, or intramuscular hemorrhage was encountered in most of the animals, and in about half there was also focal epicardial or interstitial cellular infiltration by lymphocytes, plasma cells, monocytes, neutrophil leucocytes, and proliferating fibroblasts in varying proportions. Focal hemorrhagic necrosis was noted in one (infected) animal.

In 9 of 12 monkeys given toxin, the lungs presented serous exudate in some alveoli, often subpleural, and in the periarterial lymphatics. In one focal hemorrhage was noted, and in two no significant lesions were recorded.

Widespread congestion was recorded

in 4 of 5 monkeys infected intraperitoneally with *S. dysenteriae*. Focal hemorrhage was present in 2 of these 4 and patchy serous alveolar exudate in one.

The splenic pulp was regarded as more or less congested in 14 of 16 monkeys (10 toxin, 6 infected), and the number of lymphocytes in the pulp seemed increased in the infected animals and in the 4- to 10-day fatalities among the toxin group. The splenic follicles usually appeared enlarged, with pale-staining centers interpreted as germinal centers. In 3 animals the centers were composed of large pale staining polygonal epithelioid cells. In 4 of 5 monkeys dying in less than 48 hours dilatation of follicle lymph clefts, karyorrhexis and phagocytosis of nuclear debris by follicular littoral cells were observed. Similar changes were usually absent in later deaths.

Irregular deposition of more or less numerous fine fat droplets in liver cells was noted in 7 of 9 monkeys which had received toxin, and in 3 of the 5 infected with organisms. Otherwise variable grades of capillary congestion were noted. No particular lobular location was noted for either the fatty degeneration or the congestion.

Fairly extensive studies of brain and spinal cord were made in 14 monkeys (ten toxin, 4 bacterial infection.) Tigrolysis and cell vacuolation were noted in a few motor nuclei in 3 monkeys of the toxin group, dubious small foci of hemorrhage in one more. In two of these first three monkeys there was some apparent cell depletion in affected nuclei and in one (pontile) some cell necrosis. Otherwise nerve cells in major cerebral, stem, and cord nuclei appeared normal. In 5 there was a sparse focal lymphocyte infiltration in leptomeninges or chorioid plexus of the lateral ventricle. Only congestion of gray substance was recorded in the first 2 of the 4 monkeys infected with *S. dysenteriae*.

Some 40 lymph nodes from 10 monkeys in the toxin group presented commonly focal hemorrhage into the sinuses, accompanied by swelling of littoral cells, sometimes monocyte or macrophage exudation, and more or less pronounced erythrophagia. Focal hemorrhage into the parenchyma was less frequent, and follicular hyperplasia or phagocytosis were absent.

Thirty lymph nodes from 5 infected monkeys were studied. Sinus and interstitial hemorrhages were less frequent and less severe, erythrophagia was not recorded, monocyte exudation and littoral cell proliferation seemed more pronounced, and follicular hyperplasia was noted in some nodes.

The colon in 5 monkeys dead on the 4th or 5th day after toxin administration presented a mucoepithelial exudate in the lumen and much mucin in the crypts. In 2 of these, focal hemorrhage in the mucosa or serosa was recorded. Three infected monkeys presented similar mucoepithelial exudates. All of 4 of this latter group presented purulent or fibrinopurulent or sanguinopurulent exudates on the serosa of large and small intestine, mesentery, and bladder. Mesentry and intestinal serosae presented only hemorrhage in the toxin group.

Focal hemorrhage and hemorrhagic islet thrombonecrosis were noted in the pancreas from one monkey of the toxin group. No lesions occurred in two from the bacterial group.

Focal hemorrhage appeared in the derma in those monkeys in which skin was sectioned.

DISCUSSION

Infection in monkeys was not accomplished by feeding even massive doses of Shiga cultures. None of the 22 strains used had been immediately iso-

lated from human cases although all were virulent for mice.

Fatal peritonitis was produced in monkeys only when as many as 20 billion Shiga bacilli were injected with mucin intraperitoneally. It is possible that these monkeys did not die of the infection, but rather from the effects of the toxin contained in, or produced by, such large numbers of Shiga bacilli. Many of the symptoms produced, and the histological findings as well, were identical with those produced by toxin given parenterally. Shiga organisms were not recovered from the monkey which died after 72 hours of survival. The one fatality occurring among those monkeys given less than 20 billion cells was associated with an abscess between layers of muscle at the site of injection. In this case, too, there was ample opportunity for toxin formation, and the spastic paralysis so typical of that caused by the toxin was also present.

Large amounts of toxin given orally (up to 2000 fatal i.v. doses) were relatively inocuous. Susceptibility to pepsin and to hydrochloric acid indicate that the toxin may be destroyed in the stomach. How much toxin reaches the intestines in an unaltered state is uncertain. Thus it cannot be concluded that it is not absorbed from the intestinal mucosa even though there is no indication that such absorption does occur. There is some evidence that it is readily absorbed from the trachea.

On the other hand, very small amounts of toxin given parenterally produced striking characteristic symptoms and lesions, among which were convulsive seizures, spastic paralysis, and hemorrhage into muscles of heart and intestine, into lymph nodes, adrenals, and the skin. Fatty degeneration was found in nearly all tissues examined. The fact that the toxin was not absorbed by the normal gastrointestinal tract of these monkeys should not be construed as proof that this toxin has no role in Shiga infections in man. The inflamed and ulcerated condition of intestinal mucosa found in the course of Shiga infection might allow absorption of enough toxin to cause serious symptoms in human patients, especially if the toxin is being constantly elaborated in the intestine. Apparently monkeys are not suitable animals in which to study bacillary dysentery due to *S. dysenteriae* (Shiga) by the methods used here.

SUMMARY

We have been unable to establish clear cut infection with the Shiga type of dysentery bacteria in monkeys. Neither have we been able to produce a characteristic syndrome by administering large amounts of Shiga toxin to monkeys by mouth.

However, very small amounts of toxin injected parenterally produce a characteristic clinical picture accompanied by constant pathological findings.

EFFECT OF α-TOCOPHEROL ON THE MUCIN ENHANCEMENT OF BACTERIAL INVASIVENESS

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Sandage and Stark,[1] using the toxin-antitoxin neutralization test technique of Friedemann,[2] recently showed that the enhancement of bacterial pathogenicity in serous cavities by gastric mucin and other substances could be explained in part on the basis of increased capillary permeability. They viewed the phenomenon as a reaction of mucin upon the host and not on the microorganism. They postulated that the introduction of mucin into a serous cavity was followed by the accumulation of serous fluid, which provided an excellent growth medium. A second result of the presence of mucin was to cause a peripheral deposition of fibrin on the surfaces of the serous cavity, thereby blocking the accumulation of circulating phagocytic cells. The results of Ercoli et al[3] and several others also showed the delay in serous cavity leukocytosis. However, mere increase in capillary permeability was not the only function of mucin since histamine, for example, also possessing a strong influence on capillary permeability, failed to act as an enhancement agent.

In our attempt to clarify the mechanism of the gastric mucin effect, two sets of observations have been made, one dealing with the antagonistic action of α-tocopherol on the enhancement phenomenon, the other concerning the action of injections of gastric mucin into animals subsequently injected with regular mucin-*Salmonella typhi* mixtures. The results are quite consistent with the permeability hypothesis.

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2. Friedemann, U. 1947, Bact. Rev. **11**: 275.
3. Ercoli, N., Lewis, M. N. and Harker, E. 1945, Proc. Soc. Exper. Biol. & Med. **59**: 273.

MATERIALS

A Vi-containing strain of *S. typhi*, 2TS, obtained from the California State Department of Health Laboratories, was used throughout this work and maintained on nutrient agar. In the presence of 0.9 ml of 5% gastric mucin, injected on the opposite side of the abdominal wall of an 18 gm AB strain mouse immediately after the bacterial suspension, 210 organisms are lethal for 50% of the mice injected in 43 hours.

The gastric mucin was prepared for injection by homogenizing in a Waring blendor 5 g Wilson No. 1701 granular mucin in 95 ml distilled water. Sterilization of the mucin was effected by heating to 125 C for 15 minutes. The pH was adjusted to 7.0 by addition of sterile sodium hydroxide.

The α-tocopherol (Merck Lot 42950) which contained 100 mg per ml (in peanut oil) was further diluted in sterile peanut oil as desired.

EXPERIMENTAL RESULTS

In the first series of experiments, of which a sample protocol is presented in table 1, groups of 5 mice were injected intraperitoneally with varying amounts of α-tocopherol followed in approximately 48 hours by the mucin-bacterial mixtures and the necessary controls. The antagonistic action of α-tocopherol on the mucin enhancement phenomenon is shown by examination of table 1, where it is clear that this substance, nontoxic and without virulence-enhancement ability by itself, can protect the animals against a lethal dose of *S. typhi*. The efficacy of administering the α-tocopherol well in advance may be seen by comparing the figures for 3 hours with 48 hours advanced injection. There is not enough data at hand as yet to determine whether there is a maximum concentration of tocopherol which

TABLE 1.—*The antagonistic influence of α-tocopherol on the enhancement of virulence by gastric mucin.*

Group	Tocopherol (mg)	Preparative interval (hours)	Mucin (0.9 ml of 5% suspension)	*S. typhi* (9.2×10^8)	Results Deaths/Total
1	10	48	+	+	1/5
2	5	48	+	+	3/5
3	2.5	48	+	+	0/5
4	1.0	48	+	+	0/5
5	10	3	+	+	5/5
6	5	3	+	+	5/5
7	2.5	3	+	+	4/5
8	1.0	3	+	+	5/5
9	10	48	+	0	0/5
10	5	48	+	0	1/5
11	2.5	48	+	0	0/5
12	1.0	48	+	0	1/5
13	10	48	0	0	0/3
14	5	48	0	0	0/3
15	2.5	48	0	0	1/3
16	1.0	48	0	0	0/3
17	10	48	0	+	0/3
18	5	48	0	+	0/3
19	2.5	48	0	+	0/3
20	1.0	48	0	+	0/3
21	Peanut oil only	48	+	+	2/3
22	Peanut oil only	48	+	0	0/3
23	0	0	+	+	5/5
24	0	0	+	0	0/5

All animals received same total volume and number of injections, made up in each case where necessary by saline.

can be used above which no antagonistic action is observed.

One-tenth mg tocopherol, suspended in 0.2 ml peanut oil, appeared to be the minimum amount of antagonist when studied in the presence of 0.9 ml of 5% mucin and 5×10^4 organisms. Table 2 presents the results of a titration of tocopherol activity.

The influence of preliminary injections of mucin on the subsequent injection of bacterial suspensions, the virulence of which has been enhanced with mucin, was next investigated.

Groups of 5 mice each were injected intraperitoneally with 1 ml of 5% mucin. About 50 hours later these animals along with the necessary control groups were injected with 0.9 ml of 5% mucin and 5.3×10^4 cells. The results are presented in table 3. It is clear that the action of the mucin in enhancing the virulence can be prevented by mucin itself if given at the correct time interval.

TABLE 2.—*Effect of varying amounts of α-tocopherol on enhancement of bacterial virulence by mucin.*

Group	Tocopherol (mg)	Results Deaths/Total (25 hours)
1	7	2/5
2	4	1/5
3	1	1/5
4	0.5	2/5
5	0.1	0/5
6	0.05	3/5
7	0.01	2/5
8	0	4/5

Tocopherol injected 43 hours before injection of mucin-bacterial mixture.
For all levels of tocopherol, controls were run on mucin, organisms, saline, peanut oil, as in table 1. All results: 0/3.

TABLE 3.—*Effect of injection of gastric mucin before injection of mixture of* S. typhi *and gastric mucin.*

Group	Preparative mucin injection (1.0 ml of 5.0%)	Preparative period	Results (Deaths/Total)	
			Mucin—*S. typhi* mixture	Saline—*S. typhi* mixture
1	+	50 hr.	0/5	
2	0	50 hr.	4/5	
3	+	50 hr.		0/5
4	+	21 hr.	4/5	
5	+	21 hr.		1/5
6	0		4/5	

DISCUSSION

The experiments reported herein were designed to test in part the hypothesis that the mechanism of action of hog gastric mucin on bacterial pathogenicity is based upon an alteration of capillary permeability. The well known action of α-tocopherol in decreasing capillary permeability was employed for this purpose, and the most reasonable interpretation of the data seems to be on the basis of an increased permeability effect

exerted by the mucin. The almost complete protection of mice against the "enhanced" *S. typhi* injection exerted by the α-tocopherol would be predicted on the basis of the data and argument presented by Sandage and Stark.[1] Allowing a sufficient time for the α-tocopherol to act, the permeability of the capillaries is established at a normal or decreased level such that the introduction of an agent, mucin, which normally sets up an inflammatory response, is unable to accomplish the accumulation of plasma elements in the peritoneal cavity.

As a result, the serous fluids do not accumulate, and the growth of the organisms is greatly retarded. The total bacterial population ultimately is handled in this case by the normal clearance mechanisms in the peritoneal cavity.

The effect of previous injections of gastric mucin can also be explained along the lines suggested by Sandage and Stark.[1] The first injection of mucin accomplishes the permeability alteration to be expected of it, but, at the same time, the effectiveness of such a reaction is nullified in the proper time interval by the deposition of fibrin in and on the walls of the peritoneal cavity which, according to Menkin, blocks the efferent lymph channels in the area. When the bacterial suspension is then injected in its vehicle of mucin, the area is already under the influence of the inflammatory elements, and an effective barrier to bacterial invasion has been established. The action of gastric mucin on bacterial virulence can at this time best be explained on the basis of an alteration by the mucin of the host's capillary permeability. It is therefore not correct to speak of enhanced bacterial virulence by mucin but of decreased natural resistance of the host.

SUMMARY

1. Injection of α-tocopherol intraperitoneally 48 hours before injecting a lethal mixture of gastric mucin and *S. typhi* protects the animals from death.

2. Injection of gastric mucin 48 hours before injecting the lethal mixture of mucin plus *S. typhi* also protects the animals.

3. The most plausible explanation of these observations is that the tocopherol by its action on capillary permeability prevents the accumulation of plasma elements in the peritoneal cavity in response to mucin injection and, in the opposite direction, limits the spread of infection from the cavity.

4. It is believed that early injection of mucin into the peritoneal cavity preceding the injection of mucin plus organisms therein acts also by preventing further exudation in and on the walls of the cavity, thus limiting the focus of infection to the site of injection.

After this paper was submitted for publication it was found that Oerskov[4] had obtained somewhat similar results with shigella infection as far as prior injections (24 hours) of mucin were concerned.

4. Oerskov, J. 1940, Ztschr. f. Immunitatsforsch. u. exper. Therap. **98**: 359.

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